[redacted] ENT COOPERATION TR[redacted]

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
KLANN, Ellen, M.
Dann, Dorfman, Herrell and Skillman
Suite 720
1601 Market Street
Philelphia, PA 19103
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (day/month/year) 24 February 2000 (24.02.00)	
Applicant's or agent's file reference	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US99/18267	11 August 1999 (11.08.99)	11 August 1998 (11.08.98)

Applicant
RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,CN,EP,IL,JP,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CR,CU,CZ,DE,DK,DM,EA,EE,ES,FI,GB,GD,GE,GH,
   GM,HR,HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,PT,
   RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   24 February 2000 (24.02.00) under No. WO 00/09726

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 15 May 2000 (15.05.00)	
International application No. PCT/US99/18267	**Applicant's or agent's file reference**
International filing date (day/month/year) 11 August 1999 (11.08.99)	**Priority date** (day/month/year) 11 August 1998 (11.08.98)
Applicant KIRBY, Edward, G. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

09 March 2000 (09.03.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Kiwa Mpay
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREA. /

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

KLANN, Ellen, M.
Dann, Dorfman, Herrell and Skillman
Suite 720
1601 Market Street
Philelphia, PA 19103
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year)
30 September 2000 (30.09.00)

Applicant's or agent's file reference

IMPORTANT NOTIFICATION

International application No.
PCT/US99/18267

International filing date (day/month/year)
11 August 1999 (11.08.99)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address

State of Nationality

State of Residence

Telephone No.

Facsimile No.

Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address

UNIVERSITY OF MALAGA
Edificio de Institutos
Universitarios
Parque Technolgico de Andalucia
E-29590 Campanillas
Spain

(Additional applicant for all designated States except the United States of America)

State of Nationality: ES

State of Residence: ES

Telephone No.

Facsimile No.

Teleprinter No.

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Kari Huynh-Khuong

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
RUT 98-0046P		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/18267	11 AUGUST 1999	11 AUGUST 1998
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet		
Applicant RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
09 MARCH 2000	26 JANUARY 2001
Name and mailing address of IPEA/US [illegible] Box PCT Washington, D.C. 20231 Facsimile No. [illegible]	Authorized officer DESAM M-FAIZ ZAGHMOUT Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No
PCT/US99/18267

I. Basis of the report

1 With regard to the elements of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-37, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of

[X] the claims:
pages 38-42, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of

[X] the drawings:
pages 1-3, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of

[X] the sequence listing part of the description:
pages 1-1, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[ ] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnisned

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

*** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No
PCT/US99/18267

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	2-6, 10-31, 38, 39	YES
	Claims	1, 7-9, 32-37, 40	NO
Inventive Step (IS)	Claims	16-17,29-30,38	YES
	Claims	1-15, 18-28, 31, 32-37 and 40	NO
Industrial Applicability (IA)	Claims	1-40	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

1. Claims 1, 7-9 lack novelty under PCT Article 33(2) as being anticipated by Temple et al (Molecular and General Genetics. 1993 Vol. 236 315-325).

The claims are directed to a plant expression cassette, which comprises a glutamine synthase coding sequence operably linked to a promoter, wherein said cassette was cloned into a binary vector.

The reference teaches the plasmid pGS100 containing alfalfa GS cDNA isolated from Medicago sativa cell culture line wherein said cDNA was cloned into a binary vector pMON316 in both sense and antisense orientation relative to the 35S cauliflower mosaic virus (CaMV) promoter and nopaline synthase (NOS) 3' transcription terminator (see paragraph 4, column 1, page 316). The sequence taught by the reference is the same as X69822 disclosed by the invention.

2. Claims 32-37, and 40 lack novelty under PCT Article 33(2) as being anticipated by Parsons et al (Bio/Technology. 1986. June issue. Vol. 4:533-536).

The claims are drawn to a transgenic plant which exhibits a growth rate, a protein concentration in gram per fresh weight in the mature leaves, chlorophyll concentration in gram fresh weight in the mature leaves, or leaf area in cm3 per leaf that is statistically significantly greater at the p=0.05 level than that of equivalent untransformed plants, reproductive unit therefrom, wherein the transgenic plant is from the genus Populus.

The reference et al teaches transgenic Populus plants which has inherited increase in a growth rate, a protein concentration in gram per fresh weight in the mature leaves, chlorophyll concentration in gram fresh weight in the mature leaves, or leaf area in cm3 per leaf that is statistically significantly greater (Continued on Supplemental Sheet.)

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION

The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): C12N 15/82, 5/04, 15/29; A01H 4/00, 5/00, 5/10 and US Cl.: 435/69.1, 468, 410, 419, 320.1; 536/23.1, 23.2, 23.6; 800/277, 290, 295, 319, 323

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):

at the p=0.05 level than that of equivalent untransformed plants. The transgenic plant of the prior art reference appears to be the same as the product claimed by applicant because they appear to possess the same functional characteristics, i.e (both are transgenic plants transformed with an exogenous gene). The production of a product by a particular process does not impart novelty or unobviousness to a product when the same product is taught by the prior art. This is particularly true when the properties of the product are not changed by the process in an expected manner. Additionally, characteristics such as the reproductive unit of said transgenic plants would be inherent in the transgenic Populus plant of the prior art.

3. Claims 1-15, 18-23, 31, 36-37 lack an inventive step under PCT Article 33 (a) as being obvious over Temple et al (Molecular and General Genetics. 1993. Vol. 236: 315-325) taken with Canton et al (Plant Molecular Biology. 1993. Vol. 22:819-829), Parsons et al (Bio/Technology. 1986. June. Vol. 4:533-538), and Smigocki et al (Patent Number: 5,496,732; Date of publication: 05 March, 1996).

Temple et al teach the plasmid pGS100 containing alfalfa GS cDNA isolated from Medicago sativa cell culture line wherein said cDNA was cloned into a binary vector pMON316 in both sense and antisense orientation relative to the 35S cauliflower mosaic virus (CaMV) promoter and nopaline synthase (NOS) 3' transcription terminator (see paragraph 4, column 1, page 316). The binary vector pTiT37ASE taught by the reference contain kanamycin resistant gene (NPTII) (first paragraph, column 2, page 316). The reference teaches a method for transformation of tobacco plant by Agrobacterium tumefaciens harboring the said vector (see section of "Plant transformation" in Materials and Methods"). However, the reference does not teach the insertion of the nucleotide sequence which encodes glutamine synthase into Populus (poplar), a woody perennial plant.

Canton et al teach an isolated nucleotide sequence encoding glutamine synthetase from Pinus sylvestris, a gymnosperm plant (see X69821, also cited in Figure 2 of page 823).

Parsons et al teach a method for transformation of poplar by Agrobacterium-mediated transformation (see second column, page 533).

Smigocki et al teach the use of pBin19 binary vector in Agrobacterium-mediated transformation of plant (column 4, line 41

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of Boxes I - VIII

It would have been obvious to one having ordinary skill at the time the invention was made to incorporate the method of transformation of poplar as taught by Parsons et al and modify by incorporating the binary vector taught by Temple et al which contains the nucleotide sequence which encodes glutamine synthetase in order to produce transgenic plants with improved ability for the reassimilation of ammonia released from a variety of metabolic pathways such as photorespiration, catabolism of amino acids and metabolism of phenylpropanoids (first paragraph, Introduction, page 315); wherein said transgenic plants are expected to have an improved nitrogen metabolism. A person of ordinary skill in the art can easily replace the source of the nucleotide sequence encoding glutamine synthase in the binary vector by inserting instead, the nucleotide sequence isolated by Canton et al which also encodes glutamine synthetase (Figure 2). The use of other binary vectors such as pBin19 is very common in this art as evidenced by the teaching of Smigocki et al (column 4, line 40). The reasonable expectation of success would have been there since the nucleotide sequence was successfully expressed in transgenic plant as taught by Temple et al, and exogenous gene was successfully expressed in poplar as taught by Parsons et al. Thus the claimed invention would have been prima facie obvious as a whole at the time it was made, especially in the absence of evidence to the contrary.

Claims 2-6, 10-31, 38-39 meet the criteria set out in PCT Article 33(2), because the prior art does not teach or fairly suggest the invention as claimed in this instant application.

Claims 16-17, 20-30, 33-35, 38-39 meet the criteria set out in PCT Article 33(3), because the prior art does not teach or fairly suggest the invention as claimed in this instant application.

Claims 1-40 meet the criteria set out in PCT Article 33(4), because the invention has the industrial applicability of producing transgenic plants with improved nitrogen metabolism.

---------- NEW CITATIONS ----------

CANTON et al. Molecular Characterization of a CDNA Clone Encoding Glutamine synthetase From a Gymnosperm, Pinus Sylvestris. Plant Molecular Biology. 1993, Vol. 22, pages 819-828, see entire conrent.

US 5,489,[illegible] SMIGOCKI et al 06 March 1996, see entire document.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/18267

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/82, 5/04, 15/29; A01H 4/00, 5/00, 5/10

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 468, 410, 419, 320.1; 536/23.1, 23.2, 23.6; 800/277, 290, 295, 319, 323

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, EAST, MEDLINE, AGRICOLA, BIOSIS, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	TEMPLE et al. Modulation of Glutamine Synthetase Gene Expression in Tobacco by the Introduction of an Alfalfa Glutamine Synthatase Gene in Sense and Antisense Orientation: Molecular and Biochemical Analysis. Mol. Gen. Genet. 1993, Vol. 236, pages,315-325, see entire document.	1 ----- 1-40
X ---- Y	PARSONS et al. Transformation of Poplar by Agrobacterium Tumefaciens. Bio/Technology. 1986. Vol. 4, pages 533-536, see entire document.	32-37 ------- 1-40
Y	LIN et al. A cDNA Sequence Encoding Glutamine Synthetase is Preferentially Expressed in Nodules of Vigna Aconitifolia. Plant Physiol. January 1995, Vol. 107, pages 279-280, see GENBANK/ Accession No. M94765.	1-40

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 22 NOVEMBER 1999	Date of mailing of the international search report 23 DEC 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer OUSAMA M-FAIZ ZAGHMOUT Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Intern ..ional application No.
PCT/US99/18267

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 468, 410, 419, 320.1; 536/23.1, 23.2, 23.6; 800/277, 290, 295, 319, 323

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/82, 5/04, 15/29, A01H 4/00, 5/00, 5/10

A1

(11) International Publication Number: WO 00/09726

(43) International Publication Date: 24 February 2000 (24.02.00)

(21) International Application Number: PCT/US99/18267

(22) International Filing Date: 11 August 1999 (11.08.99)

(30) Priority Data:
60/096,032 11 August 1998 (11.08.98) US

(71) Applicant *(for all designated States except US)*: RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY [US/US]; Old Queens, Somerset Street, New Brunswick, NJ 08903 (US).

(72) Inventors; and
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(74) Agents: KLANN, Ellen, M. et al.; Dann, Dorfman, Herrell and Skillman, Suite 720, 1601 Market Street, Philelphia, PA 19103 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: TRANSGENIC TREES HAVING IMPROVED NITROGEN METABOLISM

(57) Abstract

Nitrogen is one of the principal factors limiting vegetative production. The present invention has improved the nitrogen metabolism in Poplar by integrating a transgene constitutively expressing a pine glutamine synthetase into the plan genome. The resulting transgenic trees exhibit higher growth rates, protein and chlorophyll contents, and leaf area than equivalent untransformed trees. It is contemplated that this approach to nitrogen improvement will be equally successful for all woody perennials. Provided with the invention is an expression casette, a vector, and a method for increase glutamine synthetase activity in woody perennials, as well as transgenic woody perennials with enhanced nitrogen metabolism and accompanying phenotype.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

TRANSGENIC TREES HAVING IMPROVED NITROGEN METABOLISM

This application claims priority to U.S. 60/096,032, filed August 11, 1998, the entirety of which is incorporated by reference herein.

FIELD OF THE INVENTION

This invention relates to the field of plant breeding, forestry, plant transformation, and mineral nutrition. More specifically, a transgenic woody perennial plant is provided, having improved nitrogen metabolism.

BACKGROUND OF THE INVENTION

Various scientific and scholarly articles are referred to in brackets throughout the specification. These articles are incorporated by reference herein to describe the state of the art to which this invention pertains.

Plant growth and biomass accumulation are dependent on the availability, absorption and assimilation of nutrients from the environment. Nitrogen is one of the principal factors limiting vegetative production. Only a few micro-organisms are capable of reducing molecular atmospheric nitrogen (N_2) to a form usable by the plant. Plants themselves are unable to do this. Although some plants can utilize N_2 through a symbiosis with certain nitrogen-reducing micro-organisms, the majority of plants obtain nitrogen by assimilating nitrate and ammonium from the soil.

In forest trees, the capacity to assimilate nitrogen is of particular significance. In communities of perennial, long-lived species, most of the nitrogen is fixed in living tissue and is not in the soil. The availability of inorganic nitrogen in the soil is a limiting factor to tree growth (Cole and Rapp, 1981, In Reiche DE (ed) Dynamic Properties of Forest Ecosystem, pp. 341-409, Cambridge University Press, Cambridge).

Poplar trees are often used in reforestation efforts, and a variety that can grow well in low nitrogen soil would be very useful. Additionally, fast-growing, high biomass poplar trees are also very valuable for fodder and industrial wood production.

Assimilation of nitrogen by plants entails the reduction of nitrate to ammonium and its incorporation into carbon skeletons. Ammonium is assimilated into organic nitrogen mainly through the reaction catalyzed by glutamine synthetase (GS; EC 6.3.1.2). The amide group from the product of the GS reaction, glutamine, is then transferred to glutamate by action of glutamate synthetase (GOGAT; EC 1.4.7.1 and 1.4.1.14). This metabolic pathway is of crucial importance, since glutamine and glutamate are the donors for the biosynthesis of major nitrogen-containing compounds, including amino acids, nucleotides, chlorophylls, polyamines, and alkaloids (Miflin and Lea, 1980, In Miflin BJ (ed) The Biochemistry of Plants Vol 5 pp. 169-202, Academic Press Inc., London).

The biochemistry and molecular biology of the GS/GOGAT cycle has been extensively studied due to the key role these enzymes play in plant growth and development (Crawford and Arst, 1993, Ann Rev Genet 27:115-146; Lam et al., 1996, Ann Rev Plant Physiol Plant Mol Biol 47: 569-593). GS is encoded by a small family of homologous nuclear genes. The enzyme is represented by two main isoenzymes: GS1 is localized in the cytosol and GS2 is a chloroplastic enzyme. Octameric GS holoenzymes also differ in their subunit compositions, GS1 is comprised of polypeptides of 38-41 kD in most plant species, whereas the size of GS2 subunit polypeptides is about 45 kD.

The physiological roles of GS1 and GS2 are now relatively well-established in angiosperms (Lea, 1997, In Dey PM, Harborne JB (eds) Plant Biochemistry, pp. 273-313, Academic Press, San Diego). In leaves, GS2 is expressed in photosynthetic tissues and is responsible

for the incorporation of ammonium from nitrate assimilation and photorespiration. GS1 is mainly expressed in vascular elements and functions to generate glutamine for nitrogen transport within the plant.

Approaches to modify levels of key enzymes involved in steps in carbon and nitrogen assimilation and primary metabolism have been considered as a means to improve vegetative growth and biomass production (Foyer and Ferrario, 1994, Biochem Soc Trans 22: 909-915, and the references therein). All the work on metabolic engineering of the nitrogen assimilation using transgenic plants has been done in annual, herbaceous species (e.g., tobacco, *Lotus corniculatus* L.). Increases in protein content and biomass production have been reported in transgenic tobacco expressing a pea GS1 gene (Coruzzi, 1995, International Application, Patent Cooperation Treaty, WO 95/09911).

However, success with constitutively-expressed GS transgenes has been unpredictable. In other reports, transgenic herbaceous plants which over-express cytosolic GS1 fail to exhibit changes in protein, chlorophyll or biomass production (Eckes et al., 1989, Mol Gen Genet 217: 263-268; Hirel et al., 1992, Plant Mol Biol 20: 207-218; Temple et al., 1993, Mol Gen Genet 236: 315-325). These discrepancies may be due to the instability of the holoenzyme in a heterologous system and/or to the different plant species used in the transformation studies.

The effects of GS over-expression may be unique in the woody perennial species, as compared to herbaceous annual species. Woody perennial species uniquely store assimilated nitrogen during periods of less favorable growth conditions, such as would occur in winter. In poplar, for example, assimilated nitrogen can reside as vegetative storage proteins (VSPs). VSPs can be mobilized to support development during active growth (Ryan and Bormann, 1982, BioScience 32: 29-32). Synthesis of seasonal VSPs in poplar is dependent on

environmental factors, including photoperiod and nitrogen availability (reviewed in Coleman, 1993, in YWC N. B. Klopfenstein M-S. Kim, M. R. Ahuja, eds, Micropropagation, Genetic Engineering, and Molecular Biology of *Populus*, Gen. Tech. Rep. RM-GTR-297, , pp 124-130, Rocky Mountain Forest and Range Experiment Station, Fort Collins). Since spring shoot growth in poplar is correlated with nitrogen recycling (Coleman et al., 1993, Plant Physiol 102: 53-59) and glutamine is the main amino acid transported in spring xylem sap (Sauter and van Cleve, 1992, Trees 7: 26-32), enhancement of nitrogen-use efficiency as a result of ectopic GS expression could affect the availability of reduced nitrogen for initiation of rapid spring growth.

The slow growth of woody perennials as compared to herbaceous annuals may also make the effect of GS over-expression on plant growth and physiology unpredictable. Because of this comparatively slow growth rate, a particular enzyme or metabolic pathway may influence plant growth and development may be quite different than that of fast-growing herbaceous plants. This may be especially true when targets for metabolic engineering are enzymes involved in assimilation and primary metabolism, therefore having wide-spread effects on plant development.

SUMMARY OF THE INVENTION

The present invention relates to the production of transgenic woody perennial plants having improved nitrogen metabolism due to expression of chimeric transgenes, comprising the coding sequence of glutamine synthetase operably linked to appropriate 5' and 3' regulatory sequences. The present invention particularly relates to altering the expression of glutamine synthetase in such plants, thereby improving numerous agronomic, economical and environmental features of the plants, such as their ability to grow on nitrogen-poor soil, or grow optimally with minimal additions of

fertilizer. Other improvements found in these transgenic plants include enhanced or novel phenotypes, such as faster growth, higher biomass production, and higher nutritional quality of fruit, seeds and foliage.

One aspect of the invention is a plant expression cassette that will alter the level and location of glutamine synthetase in plants. This expression cassette comprises a glutamine synthetase gene operably linked to a promoter. In preferred embodiments, the glutamine synthetase gene is from a gymnosperm, the genus *Pinus*, and the species *Pinus sylvestris*. In other preferred embodiments, the expression cassette additionally comprises the cauliflower mosaic virus 35S promoter and the NOS terminator. In other preferred embodiments, the expression cassette comprises a sequence that is at least 70% identical to Genbank Accession No. X69822, encodes a protein that is at least 70% similar to Genbank Accession No. X69822, hybridized to Genbank Accession No. X69822 at moderate stringency, or is Genbank Accession No. X69822.

Another aspect of the invention is a vector containing the expression cassette. In preferred embodiments, the vector is an *Agrobacterium* binary vector and pBIN19. In another preferred embodiment, the vector comprises the neomycin phosphotransferase II coding sequence.

Another aspect of the invention is a method for producing a transgenic plant with improved nitrogen metabolism by transforming a plant *in vitro* with the aforementioned expression cassette. In preferred embodiments, the plant is a woody perennial, in the family *Salicaceae*, in the genus *Populus*, a hybrid *Populus tremula* X *P. alba*, and clone INRA 717 1-B4 of the hybrid *Populus tremula* X *P. alba*. In other preferred embodiments, the method uses *Agrobacterium tumefaciens* and the *Agrobacterium* binary vector containing the glutamine synthetase expression cassette. This aspect

includes a transgenic plant made by the method and a reproductive unit from the plant.

Another aspect of the invention is a transgenic woody perennial plant with improved nitrogen metablism which comprises at least one transgene expressing the coding sequence of glutamine synthetase. In preferred embodiments, the glutamine synthetase gene is from a gymnosperm, from *Pinus sylvestris*, and is Genbank Accession No. X69822. In other preferred embodiments, the transgenic plant is in the family Salicaceae, the genus Populus, is a hybrid *Populus tremula* X *P. alba*, and is clone INRA 717 1-B4 of the hybrid *Populus tremula* X *P. alba*. This aspect additionally includes a reproductive unit from the transgenic plant.

Another aspect of the invention is a transgenic woody perennial that exhibits a growth rate over the first two months in the greenhouse that is at least XX% greater than that of equivalent untransformed plants. In a preferred embodiment, the plant additionally exhibits a protein concentration (g/gfw) that is at least 10% greater than that of equivalent untransformed plants after the first XX months in the greenhouse. In a most preferred embodiment, the transgenic plant additionally exhibits a chlorophyll concentration (g/gfw) that is at least 10% greater than that of equivalent untransformed plants after the first XX months in the greenhouse. In other preferred embodiments, the plant is in the family Salicaceae, in the genus Populus, a hybrid of *Populus tremula* X *P. alba*, and is clone INRA 717 1-B4 of the hybrid *Populus tremula* X *P. alba*. This aspect additionally contains a reproductive unit of the transgenic plant.

Other features and advantages of the present invention will be better understood by reference to the drawings, detailed description and examples that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Strategy for transformation of hybrid poplar (*Populus tremula X P. alba* [INRA 717 1-B] with *Agrobacterium tumefaciens* (LBA 4404) containing the binary vector pBin35SGSp. B/E* represents a blunt-ended *Bam*HI/*Eco*RI site. The arrows in pBin35SGSp indicate the sense of transcription of CaMV 35S-GS-NOS and *nptII* genes.

Figure 2. The average height of controls (column 1) and transgenic trees (column 2) 2 months after the plants were moved to the greenhouse. Columns 3-24 represent the 22 transgenic lines. The height of a total of 5 controls and 78 transgenic trees were measured after 2 months in the greenhouse. The net average height of the 22 transgenic lines after 2 months in the greenhouse was 76% higher than the controls.

Figure 3. Net height of control and transgenic plants during the first 6 months in greenhouse. The heights of a total of 5 controls and 78 transgenic plants were measured weekly. Square data points, average height of 22 transgenic lines; diamond data points, line 8-5, one of the fastest growth transgenic lines; and triangular data points, average height of 5 control plants.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

Various terms relating to the methods and compositions of the present invention are used hereinabove and also throughout the specifications and claims.

Various terms relating to the biological molecules of the present invention are used hereinabove and also throughout the specifications and claims. The terms "substantially the same," "percent similarity" and

"percent identity" are defined in detail below.

With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to genomic DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryote or eukaryote. An "isolated nucleic acid molecule" may also comprise a cDNA molecule or a synthetic DNA molecule.

With respect to RNA molecules of the invention, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e., in cells or tissues), such that it exists in a "substantially pure" form .

Nucleic acid sequences and amino acid sequences can be compared using computer programs that align the similar sequences of the nucleic or amino acids thus define the differences. For purposes of this invention, the GCG Wisconsin Package version 9.1, available from the Genetics Computer Group in Madison, Wisconsin, and the default parameters used (gap creation penalty=12, gap extension penalty=4) by that program are the parameters intended to be used herein to compare sequence identity and similarity.

The term "substantially the same" refers to nucleic acid or amino acid sequences having sequence variations that do not materially affect the nature of the protein (i.e. the structure, thermostability characteristics and/or biological activity of the protein). With particular reference to nucleic acid

sequences, the term "substantially the same" is intended to refer to the coding region and to conserved sequences governing expression, and refers primarily to degenerate codons encoding the same amino acid, or alternate codons encoding conservative substitute amino acids in the encoded polypeptide. With reference to amino acid sequences, the term "substantially the same" refers generally to conservative substitutions and/or variations in regions of the polypeptide not involved in determination of structure or function.

The terms "percent identical" and "percent similar" are also used herein in comparisons among amino acid and nucleic acid sequences. When referring to amino acid sequences, "percent identical" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical amino acids in the compared amino acid sequence by a sequence analysis program. "Percent similar" refers to the percent of the amino acids of the subject amino acid sequence that have been matched to identical or conserved amino acids. Conserved amino acids are those which differ in structure but are similar in physical properties such that the exchange of one for another would not appreciably change the tertiary structure of the resulting protein. Conservative substitutions are defined in Taylor (1986, J. Theor. Biol. 119:205). When referring to nucleic acid molecules, "percent identical" refers to the percent of the nucleotides of the subject nucleic acid sequence that have been matched to identical nucleotides by a sequence analysis program.

The term "ectopic expression" refers to a pattern of subcellular, cell-type, tissue-type and/or developmental or temporal(e.g., light/dark) expression that is not normal for the particular gene or enzyme in question. Such ectopic expression does not necessarily exclude expression in normal tissues or developmental stages.

The term "overexpression" means a greater than

normal expression level of a gene in the particular tissue, cell and/or developmental or temporal stage for the gene. Such overexpression results in "overproduction" of the enzyme encoded by the gene, which means a greater than normal production of the enzyme in a particular tissue or cell, or developmental or temporal stage for the enzyme. The terms "underexpression" and "underproduction" have an analogously converse meaning, and are used interchangeably with the term "suppression".

In regards to the present invention, "equivalent plants" are ones of the same genotype or cultivar, at the same age, and having been grown under the same conditions. In the case where one is a transgenic plant, the equivalent plant may be transformed by a similar DNA construct but without the glutamine synthetase transgene, or may not be transformed but regenerated from tissue culture.

In this invention, the term "promoter" or "promoter region" refers to the 5' regulatory regions of a gene, including promoters *per se* (e.g., CaMV 35S promoters and/or tetracycline repressor/operator gene promoters), as well as other transcriptional and translational regulatory sequences.

The term "selectable marker" refers to a gene product that confers a selectable phenotype, such as antibiotic resistance, on a transformed cell or plant. Selectable markers are encoded by expressible DNA sequences, which are sometimes referred to herein as "selectable marker genes."

The terms "operably linked", "operably inserted" or "operably associated" mean that the regulatory sequences necessary for expression of the coding sequences are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of coding sequences and transcription control elements (e.g. promoters, enhancers, and termination elements) in

an expression vector.

The term "DNA construct" refers to genetic sequence used to transform plant cells and generate progeny transgenic plants. At minimum a DNA construct comprises a coding region for a selected gene product, operably linked to 5' and 3' regulatory sequences for expression in transformed plants. In preferred embodiments, such constructs are chimeric, i.e., the coding sequence is from a different source one or more of the regulatory sequences (e.g., coding sequence from tobacco and promoter from cauliflower mosaic virus). However, non-chimeric DNA constructs also can be used.

DNA constructs may be administered to plants in a viral or plasmid vector. Other methods of delivery such as *Agrobacterium* T-DNA mediated transformation and transformation using the biolistic process are also contemplated to be within the scope of the present invention. The transforming DNA may be prepared according to standard protocols such as those set forth in Ausubel et al. (1998). A plant species or cultivar may be transformed with a DNA construct (chimeric or non-chimeric) that encodes a polypeptide from a different plant species or cultivar (e.g., poplar transformed with a gene encoding a pine protein). Alternatively, a plant species or cultivar may be transformed with a DNA construct (chimeric or non-chimeric) that encodes a polypeptide from the same plant species or cultivar. The term "transgene" is sometimes used to refer to the DNA construct within the transformed cell or plant.

In accordance with the present invention, nucleic acids having the appropriate sequence homology with the nucleic acids of the invention may be identified by using hybridization and washing conditions of appropriate stringency. For example, hybridizations may be performed, according to the method of Sambrook et al. (1989, Molecular Cloning, Cold Spring Harbor Laboratory), using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 µg/ml denatured,

fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules of a specified sequence homology (Sambrook et al., 1989, *supra*):

$$T_m = 81.5°C + 16.6\text{Log}[Na+] + 0.41(\%\ G+C) - 0.63\ (\%\ \text{formamide}) - 600/\#\text{bp in duplex}$$

As an illustration of the above formula, using [Na+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of 42°C.

The stringency of the hybridization and wash depend primarily on the salt concentration and temperature of the solutions. In general, to maximize the rate of annealing of the probe with its target, the hybridization is usually carried out at salt and temperature conditions that are 20-25°C below the calculated T_m of the hybrid. Wash conditions should be as stringent as possible for the degree of identity of the probe for the target. In general, wash conditions are selected to be approximately 12-20°C below the T_m of the hybrid. In regards to the nucleic acids of the current invention, a moderate stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and washed in 2X SSC and 0.5% SDS at 55°C for 15 minutes. A

high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and washed in 1X SSC and 0.5% SDS at 65°C for 15 minutes. A very high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and washed in 0.1X SSC and 0.5% SDS at 65°C for 15 minutes.

II. Description

The present invention provides a transgenic woody perennial plant with altered nitrogen assimilation and/or utilization. In particular, the invention relates to altering the activity of enzymes involved in nitrogen assimilation and utilization in order to engineer trees with better growth characteristics, higher biomass production, less requirement for fertilizer, better nutritional qualities, and/or improved seed or fruit yield.

A particularly preferred embodiment of the invention, which is described in greater detail in Example 1, comprises poplar trees engineered to ectopically over-express glutamine synthetase (GS). Poplar was transformed with a heterologous GS1 (cytosolic) gene from pine. All plants regenerated in presence of the antibiotic were shown to contain the pine GS1 gene. Pine GS1 transgene expression was also detected in all selected poplar lines, and high levels of pine GS1 mRNA were shown in leaf tissues of transgenic plants. The pine GS1 polypeptide was also detected both in leaf regions enriched in photosynthetic cells (leaf blades) and in vascular elements (petioles), indicating that the gymnosperm GS1 transcripts are correctly processed by the angiosperm translational machinery. It is worthy of particular note that cytosolic GS expression in angiosperm leaves is confined to vascular elements, and therefore pine GS is ectopically expressed in poplar.

The presence of the pine GS1 polypeptide in young leaves of transgenic poplar suggests firstly that the pine GS1 protein is stable in photosynthetic cells. Moreover, ectopic expression of the pine GS1 in transgenic poplar leads to an increase in the capacity for ammonia assimilation and glutamine biosynthesis. These findings demonstrate that pine GS1 subunits are assembled in functional holoenzymes in most if not all tissues of transgenic poplar, even tissues where the endogenous cytosolic GS is not normally expressed.

The above-noted changes in GS1 expression in poplar are accompanied by modification in phenotype, including increased growth and increased leaf dimensions. The increase in GS activity in transgenic poplar leaves is positively correlated with increased protein and chlorophyll accumulation. These results show that over-expression of a cytosolic GS gene has a global effect on the synthesis of nitrogen-containing molecules and, therefore, produces a global effect on plant development.

The experimental results described hereinabove in accordance with the present invention are the first to demonstrate the increase in nitrogen-use efficiency in transgenic trees by overproducing a key enzyme involved in nitrogen metabolism. These results are surprising and unexpected, for several reasons. First, the transformation efficiency is unexpectedly high, nearly 100%, as compared with 15-20% efficiency for similar transformations reported in other woody plants. Secondly, the unusual accumulation the pine GS1 in photosynthetic tissue of poplar could not have been predicted from the normal accumulation of the endogenous angiosperm enzyme in vascular tissue only. While not limiting the mechanism of the invention in any one explanation, this unusual pine GS1 localization may be contributing to the novel phenotypic effects of the transgene as explained below.

Most significantly, however, the plant-wide improvement in growth rate and productivity observed in

these trees as a result of altering the glutamine synthetase expression is surprising and unexpected. Without being limited by any particular explanation of these results, the expression of a gymnosperm GS1-encoding gene in an angiosperm cellular environment may play a significant role in the improved nitrogen metabolism and resultant phenotypes observed. For instance, the heterologous GS1 gene or its encoded enzyme may circumvent possible down-regulatory mechanisms affecting the endogenous GS enzymes. Another possibility is that the gymnosperm enzyme may be better expressed, more stable or intrinsically more efficient than the angiosperm protein. This is likely in view of the fact that, in gymnosperms, the GS2 chloroplastic enzyme is not expressed, so the GS1 enzyme may have evolved to compensate, e.g., by higher gene expression, improved mRNA stability, improved protein stability or increased activity. The use of a transgene containing the gymnosperm GS1 enzyme in angiosperm plants represents a new a novel approach to the improvement of nitrogen metabolism.

Provided in accordance with the present invention is an expression cassette for altering the level of glutamine synthetase in plant cells. The expression cassette can be used to manipulate nitrogen metabolism in plants. In a preferred embodiment, the expression cassette comprises the coding sequence of a gymnosperm glutamine synthetase gene operably linked to a promoter. While the use of the *Pinus sylvestris* glutamine synthetase gene coding sequence is taught in Example 1, it is contemplated that any gymnosperm glutamine synthetase gene can be used to achieve similar results. In a more preferred embodiment, a *Pinus* glutamine synthetase gene coding sequence is used. In a most preferred embodiment, a glutamine synthetase gene from *Pinus sylvestris* is used.

In another preferred embodiments, the expression cassette contains sequences that are similar

to the to the pine GS1 coding sequence. Because each amino acid is encoded by several codons, a protein identical to *Pinus sylvestris* GS1 may be encoded by many different coding sequences. Additionally, proteins have a similar enzymatic function to GS1 and yet have a different amino acid sequence through the substitution of structurally similar amino acids. Therefore coding sequences that are similar yet not identical to *Pinus sylvestris* GS1 are contemplated in regards to the present invention. In a preferred embodiment, the expression vector comprises a nucleic acid sequence is at least 70% identical to Genbank Accession No. X69822. The nucleic acid sequences are at least 80% identical in a more preferred embodiment, and at least 90% identical in a most preferred embodiment. In another embodiment, the expression cassette contains a coding sequence which encodes a protein that is at least 70% similar to Genbank Accession No. X69822. The sequence encodes a amino acid sequence that is at least 80% similar in a more preferred embodiment, and at least 90% similar in a most preferred embodiment. In another embodiment, the expression cassette hybridizes to the nucleic acid in Genbank Accession No. X69822 under conditions of moderate stringency in a preferred embodiment, high stringency in a more preferred embodiment, and very high stringency in most preferred embodiment.

Expression cassettes for expressing a DNA sequences in selected plant cells comprise a DNA sequence of interest operably linked to appropriate 5' (e.g., promoters and translational regulatory sequences) and 3' regulatory sequences (e.g., terminators). In a preferred embodiment, the coding region of a gymnosperm glutamine synthetase gene is placed under a powerful constitutive promoter, such as the Cauliflower Mosaic Virus (CaMV) 35S promoter. Other constitutive promoters contemplated for use in the present invention include, but are not limited to: figwort mosaic virus 35S promoter, T-DNA mannopine synthetase, nopaline synthase (NOS) and octopine synthase

(OCS) promoters.

Expression cassettes that express a gymnosperm glutamine synthetase gene coding sequence under an inducible promoter (either its own promoter or a heterologous promoter) are also contemplated to be within the scope of the present invention. Inducible plant promoters include the tetracycline repressor/operator controlled promoter, the heat shock gene promoters, stress (e.g., wounding)-induced promoters, defense responsive gene promoters (e.g. phenylalanine ammonia lyase genes), wound induced gene promoters (e.g. hydroxyproline rich cell wall protein genes), chemically-inducible gene promoters (e.g., nitrate reductase genes, gluconase genes, chitinase genes, etc.) and dark-inducible gene promoters (e.g., asparagine synthetase gene) to name a few.

Organelle-specific, tissue-specific, and development-specific promoters are also contemplated for use in the present invention. Examples of these included, but are not limited to: the ribulose bisphosphate carboxylase (RuBisCo) small subunit gene promoters or chlorophyll a/b binding protein (CAB) gene promoters for expression in photosynthetic tissue; the various seed storage protein gene promoters for expression in seeds; and the root-specific glutamine synthetase gene promoters where expression in roots is desired. Examples of organelle specific promoters include, but are not limited to the ribulose bisphosphate carboxylase (RuBisCo) large subunit gene promoter and the D1 protein promoter. In a preferred embodiment, the expression cassette comprises a chloroplast specific promoter.

Expression cassettes that down-regulate or inhibit expression of glutamine synthetase are also contemplated in accordance with the present invention. This may be necessary in order to divert nitrogen assimilation or utilization to an alternative pathway, e.g., an engineered pathway that is more efficient than

the natural pathway. To accomplish this, the gymnosperm glutamine synthetase gene coding sequence or a fragment thereof may be utilized to control the production of the encoded protein. In one embodiment, full-length antisense molecules or antisense oligonucleotides, targeted to specific regions of the encoded RNA that are critical for translation, are used. The use of antisense molecules to decrease expression levels of a pre-determined gene is known in the art. In a preferred embodiment, the expression cassette expresses all or part of the antisense strand of a glutamine synthetase gene coding sequence. In another embodiment, an expression cassette that causes the over-expression of the gene targeted for down-regulation is induced to generate a co-suppression effect. In another embodiment, an expression cassette for down-regulation of the GS enzyme comprises a sequence that encodes a GS with mutations in the active site of enzyme.

In some instances, it may be advantageous to engineer the expression cassette such that it encodes a "transit" sequence enabling the encoded glutamine synthetase to cross the chloroplast membrane and localize within the chloroplast. The chloroplastic GS2 naturally comprises such a transit sequence. Cytosolic isozymes, such as GS1, can be targeted to the chloroplast through the in-frame inclusion of a DNA segment encoding such a transit sequence, according to known methods. This expression cassette may be of particular utility in production of transgenic gymnosperms having improved nitrogen metabolism, given that the gymnosperm chloroplastic GS2 is not expressed.

The coding region of the expression cassette is also operably linked to an appropriate 3' regulatory sequence. In a preferred embodiment, the nopaline synthetase polyadenylation region (NOS) is used. Other useful 3' regulatory regions include, but are not limited to the octopine (OCS) polyadenylation region.

Also provided in accordance with the present

invention is a vector containing the expression cassette of the invention. This vector may be used to maintain the expression cassette in bacteria, such as *Echerichia coli*. Vectors that may be used to maintain the expression cassette in *E. coli* are well known to those in the art. The expression cassette may also have a more specialized function of introducing the expression cassette into a plant cell. These vectors may be specialized for the various well known ways of introducing transgenes into plant cells. Vectors that may be used for chloroplast transformation are contemplated in regards to the present invention. Examples of vectors for chloroplast transformation include, but are not limited to, pZS197 (Svab and Maliga, 1993, PNAS 90:915-917). In a most preferred embodiment, the vector contains the nucleic acid sequences needed to allow the expression cassette to be stably inserted into the genome of the desired woody perennial by *Agrobacterium tumefaciens*-mediated plant transformation.

In a preferred embodiment, the vector is an *Agrobacterium* binary vector. Such vectors include, but are not limited to, BIN19 (Bevan, 1984, Nucleic Acid Res 12: 8711-8721) and derivatives thereof, the pBI vector series (Jefferson et al., 1987, PNAS 83:8447-51), and binary vectors pGA482 and pGA492 (An, 1986) and others (for review, see An, 1995, Methods Mol Biol 44:47-58). In a particularly preferred embodiment, the vector is pBIN19 (Bevan, 1984, Nucleic Acid Res 12: 8711-8721).

Using an *Agrobacterium* binary vector system, the aforementioned expression cassette is linked to a nuclear drug resistance marker, such as kanamycin. In a preferred embodiment, the neomycin phosphotransferase II gene from pCaMVNEO is used (Fromm et al., 1986, Nature 319: 791-793). Other useful selectable marker systems include, but are not limited to: other genes that confer antibiotic resistances (e.g., resistance to hygromycin or bialaphos) or herbicide resistance (e.g., resistance to sulfonylurea, phosphinothricin or glyphosate). In a most

preferred embodiment, the vector is that shown in Figure 1.

Also provided in accordance with the present is a method to make a woody perennial plant with altered concentrations of glutamine synthetase in its cells. This method comprises the step of stabily integrating the expression cassette with THE gymnosperm glutamine synthetase coding sequence into the genome of a woody perennial plant cell. Several ways to integrate a transgene such as the expression cassette into a plant cell genome are possible, including but limited to, *Agrobacterium* vectors, PEG treatment of protoplasts, biolistic DNA delivery, UV laser microbeam, gemini virus vectors, calcium phosphate treatment of protoplasts, electroporation of isolated protoplasts, agitation of cell suspensions with microbeads coated with the transforming DNA, direct DNA uptake, liposome-mediated DNA uptake and chloroplast transformation (Maliga et al., 1995, U.S. Patent 5,451,513). Such methods have been published in the art. See, e.g., Methods for Plant Molecular Biology (Weissbach & Weissbach, eds., 1988); Methods in Plant Molecular Biology (Schuler & Zielinski, eds., 1989); Plant Molecular Biology Manual (Gelvin, Schilperoort, Verma, eds., 1993); and Methods in Plant Molecular Biology - A Laboratory Manual (Maliga, Klessig, Cashmore, Gruissem & Varner, eds., 1994). In a preferred embodiment, *Agrobacterium*-mediated transformation is used.

Agrobacterium-mediated transformation of plant nuclei is accomplished according to the following procedure:

(1) the gene is inserted into the selected *Agrobacterium* binary vector;

(2) transformation is accomplished by co-cultivation of an appropriate plant tissue (such as leaf tissue in poplar) with a suspension of recombinant *Agrobacterium*, followed by incubation (e.g., two days) on growth medium in the absence of the drug used as the

selective medium (see, e.g., Horsch et al., 1985, Cold Spring Harb Symp Quant Biol 50:433-7);

(3) plant tissue is then transferred onto the selective medium to identify transformed tissue; and

(4) identified transformants are regenerated to intact plants.

It should be recognized that the amount of expression, as well as the tissue specificity of expression of the transgenes in transformed plants can vary depending on the position of their insertion into the nuclear genome. Such position effects are well known in the art. For this reason, several transformants should be regenerated and tested for expression of the transgene.

Plants are transformed and thereafter screened for one or more properties, including expression of the transgene, altered nitrogen assimilation and/or utilization capacities, higher growth rates, biomass accumulation rates, higher protein or chlorophyll concentration, or changes in growth habit or appearance (e.g., alteration of phyllotaxy and canopy structure - the arrangement of leaves and branches to optimize light reception - alterations of which have been observed in the exemplified transgenic poplar).

Also provided in accordance with the present invention is woody perennial plant with altered concentrations of glutamine synthetase in its cells, which exhibits altered nitrogen metabolism. The successful transformation of poplar (an angiosperm) with a pine (a gymnosperm) GS1 gene, and the greatly improved phenotype obtained thereby, indicates that nitrogen metabolism may be improved in woody perennials more dramatically than hitherto expected. Accordingly, although in a particularly preferred embodiment the woody perennial is poplar, (specifically hybrid poplar clone INRA 7171-B4, *Populus tremula* X *P. alba*), other members of the genus *Populus* (which includes cottonwood, aspen and poplar) and the family Salicaceae are also preferred

for practice of the present invention. In other embodiments, a wide variety of woody perennials are contemplated as targets for similar genetic engineering using the compositions and methods described herein. These include, but are not limited to, angiosperm forest trees, such as eucalyptus, willow (*Salix spp.*), birch, oak, cherry, maple, yellow or tulip poplar (genus *Liriodendron*), sweetgum, acacia, teak, *Liquidamber spp.* and *Alnus spp.*, among others; gymnosperm forest trees, such as pine, spruce, fir, redwood, Douglas fir, *Araucaria spp.* and *Cryptomeria spp.*, among others; as well as fruit and nut-bearing trees and ornamental trees and shrubs.

Also provided in accordance with the current invention is a poplar tree that has a statistically significant higher growth rate, higher protein and chlorophyll content in mature leaves, and larger mature leaf dimensions than its untransformed equivalent. In a preferred embodiment, this transgenic tree exhibits at least 10% greater growth rate during the first 3 months in the greenhouse after transformation as compared to untransformed trees of the same cultivar. More preferably, the transgenic poplar is 40% greater, and in a most preferred embodiment, the transgenic tree is 60% greater. In a more preferred embodiment, the transgenic poplar additionally has at least 10% greater grams of protein in the leaf tissue at XX months per gram fresh weight as compared to untransformed trees of the same cultivar. More preferably, the tree exhibits at least 15% greater protein, and most preferably, the tree exhibits at least 25% greater protein per gram per gram fresh weight. In a particularly preferred embodiment, the transgenic poplar additionally has at least 10% greater grams of chlorophyll per gram fresh weight in mature leaf tissue as compared to control trees of the same cultivar. In a more preferred embodiment, the trees have at least 15% greater chlorophyll, and in a most preferred embodiment, the trees have at least 20% greater

chlorophyll per gram per gram fresh weight. In a more particularly preferred embodiment, the transgenic poplar additionally has at least 10% greater area per mature leaf as compared to control trees of the same cultivar. In a more preferred embodiment, the trees have at least 15% greater leaf area, and in a most preferred embodiment, the trees have at least 20% greater leaf area per leaf. In regards to the present invention, statistical significance of quantified differences is determined using one-way analysis of variance (ANOVA). This statistical test is well known to those in the art, and computer programs that carry out this test are commercially available. The level of probably (P) used is 0.05 in a preferred embodiment, 0.01 in a more preferred embodiment, and 0.001 in a most preferred embodiment.

The preceding description set forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general cloning procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989) or Ausubel et al. (eds.) Current Protocols in Molecular Biology, John Wiley & Sons (1999) are used.

III. Uses for the Woody Perennials with Altered Nitrogen Metabolism

The genetically modified trees and other woody perennial plants of the present invention are expected to be of use for a variety of agronomic and/or horticultural purposes. For instance, due to their improved nitrogen metabolism, they may be productively cultivated under nitrogen nutrient deficient conditions (i.e., nitrogen-poor soils and low nitrogen fertilizer inputs) that would be detrimental to the growth of wild-type trees. The engineered trees may also be advantageously used to

achieve earlier maturing, faster growing, and/or higher yielding crops and/or produce more nutritious foods (fruit and nuts) and animal fodder when cultivated under nitrogen non-limiting growth conditions (i.e. soils or media containing or receiving sufficient amounts of nitrogen nutrients to sustain healthy tree growth).

The trees and other woody plants of the present invention also have utility as improved ornamental varieties. In addition to faster growth, these trees may have improved canopy characteristics (e.g., as results from altered phyllotaxy as discussed above), higher concentrations of pigment molecules or higher chlorophyll contents.

The trees and other woody plants of the present invention additionally have utility for forestry. Fast growing, high biomass trees have great economic value to the lumber and paper industries. The fast growing and nitrogen efficient trees of the present invention are also extremely useful for reforestation efforts, where soil nitrogen is often limiting and vigorous growth essential. Improved nitrogen assimilation also may result in higher concentrations of useful nitrogen-containing secondary compounds, such as the alkaloid quinine, or compounds involved in resistance to insects or pathogenic microorganisms.

The transgenic plants of the invention may be used for plant breeding or directly in silvaculture applications. Plants containing one transgene may be crossed with plants containing a complementary transgene in order to produce plants with enhanced or combined phenotypes.

The following example is provided to describe the invention in greater detail. It is intended to illustrate, not to limit, the invention.

EXAMPLE I
Transformation of Poplar with a Chimeric Glutamine Synthetase Gene

This example describes the transformation of poplar with a synthetic glutamine synthetase gene.

Materials and Methods

Plant materials. Hybrid poplar (*Populus tremula* X *P. alba*. clone INRA 717 1-B4) was maintained *in vitro*, as described (Leple et al., 1992, Plant Cell Reports 11: 137-141). Clone INRA 717 1-B4 was obtained from Dr. Lise Jouanin, Cell Biology Laboratory, INRA, Versailles, FRANCE 78026.

Gene construction. A chimeric gene composed of the cauliflower mosaic virus (CaMV) 35S promoter fused to the pine cytosolic glutamine synthetase (GS) cDNA (Cantón et al., 1993, Plant Mol Biol 22: 819-828; Genbank Accession No. X69822) and nopaline synthetase polyadenylation region (NOS) was used to transform hybrid poplar (Figure 1). The 1.4 kb *EcoRI* insert containing the full-length cytosolic GS cDNA from pGS114 (Cantón et al., 1993, Plant Mol Biol 22: 819-828) was isolated and blunt ended using the Klenow fragment of DNA polymerase I. In parallel, the 1.0 kb *BamHI* fragment containing the neomycin phosphotransferase II (NPTII) gene from pCaMVNEO (Fromm et al., 1986, Nature 319: 791-793) was excised and the digested plasmid was blunt-ended. The 1.4 kb GS cDNA was then ligated into the digested pCaMVNEO to yield p35SGSp. The new plasmid has a 2.1 kb *HindIII* fragment containing the CaMV 35S-GS-NOS construct (Figure 1). The orientation of the GS cDNA was verified by sequencing the junctions. This 2.1 kb *HindIII* construct was then ligated into the *HindIII* site of the *Ti*-derived disarmed binary vector pBin19 (Bevan, 1984, Nucleic Acid Res 12: 8711-8721). The new vector, pBin35SGSp, was transferred into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method (Holsters et al., 1978, Mol Gen Genet

163:181-187).

Preparation of Agrobacterium. A single colony of *Agrobacterium tumefaciens* strain LBA4404 containing the binary plasmid vector, pBin35SGSp, as described above, was cultured in 2YT (Ausubel et al., 1987, Current Protocols in Molecular Biology. Wiley Interscience, New York) liquid medium containing antibiotics: streptomycin (200 mg L^{-1}) and kanamycin (50 mg L^{-1}). After 48 hours at 28° C (300 rpm), the bacterial suspension was centrifuged and bacteria resuspended in liquid M1 plant cell culture medium (see below) to an OD_{660} of 0.3.

Inoculation, co-cultivation, decontamination, selection, and regeneration. When *in vitro* grown plantlets reached a height of 5-10 cm, leaves were removed and pre-cultured in darkness for 48 hours on solidified M1 medium consisting of MS salts (Murashige and Skoog, 1962, Physiol Plant 15: 473-497), MW vitamins (Morel and Wetmore, 1951, Am J Bot 38: 141-143), 3% (w/v) sucrose, L-cysteine (1 mg L^{-1}) and Bacto-agar (8 g L^{-1}). Pretreated leaves were cut into segments 1 cm X 1 cm. Leaf segments were placed directly in bacterial suspension at room temperature for 2 h then blotted onto sterile filter paper to remove excess bacteria. Explants were co-cultivated in darkness for 48 h on solidified M1 medium. For decontamination and selection for antibiotic resistance, explants were transferred to M2 medium, (consisting of M1 medium containing timentin (200 mg L^{-1}), kanamycin (50 mg L^{-1}), and 2,4-D (1 mg L^{-1})) in darkness. After 4 weeks calli were separated from leaf segments and transferred to M3 medium, (consisting of M1 medium containing kanamycin (50 mg L^{-1}) and thidiazuron (0.1 mM)) in the light for regeneration of shoots. After shoots reached a height of 2-3 cm, they were separated and cultured on M4 medium (consisting of M1 with half-strength MS macronutrients) for root induction.

Plant culture conditions. Unless otherwise noted, *in vitro* cultures were maintained in a constant temperature facility at 24° C and provided with low light

(30 mmol m^{-2} sec^{-1}; 16 h photoperiod, General Electric Cool-White fluorescent bulbs). After roots were induced, rooted shoots of transformed and control plants were transferred to a potting mix consisting of MetroMix 200 (Scotts Co., Marysville Ohio USA). Plants were supplied with water-soluble fertilizer (20:20:20) every six weeks.

Protein extraction. Measuring from the apex, the fourth leaf of 3-4 month old greenhouse-grown plants were ground in a mortar with a pestle using glass beads (Sigma) in 50 mM Tris-HCl, 2 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol (v/v), pH 8.0. The ratio of extraction buffer to glass beads to plant material was 3:0.5:1. Extracts were centrifuged at 22,000 x g and the supernatant used for GS activity and protein determinations.

Electrophoresis and immunoblot detection of GS polypeptides. Total soluble proteins were separated by SDS-PAGE (10% acrylamide) using the discontinuous buffer system of Laemmli (Laemmli, 1970, Nature 227:680-685). Polypeptides were visualized by Coomassie-blue staining to confirm that equal amounts of protein were loaded in each lane. Proteins were electro-transferred to nylon filters and GS polypeptides were detected using polyclonal antibodies raised against the recombinant pine GS (Cantón et al., 1996, FEBS Lett 393: 205-210). Protein blotting, saturation of blot, and subsequent incubations with the antiserum and washing steps were performed as described elsewhere (Gallardo et al., 1995, Planta 197: 324-332). Immuno-complexes were detected with peroxidase-conjugated immunoglobulin with a molar ratio of peroxidase to immunoglobulin of 3.3 (Vector Laboratories, Burlingame, CA).

Nucleic acid isolation and analysis. Genomic DNA was isolated from poplar leaves according to Dellaporta et al. (Dellaporta et al., 1993, Plant Mol Biol Rep 4:19-21). Total RNA was extracted from 5 g of leaves using the guanidine thiocyanate method as described elsewhere (Ausubel et al., 1987, Current

Protocols in Molecular Biology. Wiley Interscience, New York). Southern and Northern blots were carried out following standard procedures (Ausubel et al., 1987, *supra*) and hybridized with single-stranded ^{32}P-labeled pine GS cDNA (1.4 kb) (Cantón et al., 1993, Plant Mol Biol 22: 819-828), made using the random primer method. A mitochondrial ß-ATP synthase probe from *Nicotiana plumbaginifolia* of 1.25 kb (Boutry and Chua, 1985, EMBO J 4:2159-2165) was used as control in Northern blot experiments.

For PCR amplification of genomic poplar DNA two specific primers, 5'-TGTTGATGCCCATTATAAGGCTTGTCTCTA-3' (SEQ ID NO:1) and 5'-GGTCGTCTCAGCAATC-AT-3' (SEQ ID NO:2), were constructed, sense and antisense sequences, respectively, of the pine GS1cDNA (Cantón et al., 1993, Plant Mol Biol 22: 819-828). The corresponding expected size of the amplification product was 538 bp. PCR reactions contained 1 µg of total genomic DNA, 20 pmol of each primer, 0.2 mM dNTP, 50 mM KCl, 10 mM Tris pH 8.3 and Taq DNA polymerase (Boehringer). Reaction mixtures were incubated in a thermocycler for 1 min at 92°C, followed by 30 cycles of 1 min at 91°C, 1 min at 45°C, 2 min at 72°C and finally 5 min at 72°C. PCR amplified fragments were analyzed by using standard protocols (Ausubel et al., 1987, *supra*).

Results

Glutamine synthetase polypeptides in leaves of hybrid poplar. In order to determine the pattern of GS polypeptides in poplar leaves, total soluble proteins were extracted from areas enriched in photosynthetic cells (leaf tip) and areas enriched in vascular tissue (petioles). Proteins were then separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with polyclonal antibodies raised against the pine glutamine synthetase (Cantón et al., 1996, FEBS Lett 393: 205-210). Photosynthetic tissues display a GS polypeptide pattern

enriched in the 45 kD polypeptide characteristic of the chloroplastic GS2, whereas vascular tissues (petioles) show a GS pattern characterized by the abundance of smaller, 40 kD polypeptide subunit of the cytosolic GS1; since these tissues are not homogenous in their cell type content, the chloroplastic and cytosolic GS polypeptides are also detected as secondary bands in the protein extracts prepared from petioles and leaf tip respectively.

Gene fusions and transformation of hybrid poplar with the pine GS1. A recombinant plasmid (pBin35SGSp) containing a chimeric GS gene construct was transferred via *Agrobacterium* to leaf segments of receptor hybrid poplar plants (Figure 1). The gene construct consisted of the 1.4 kb pine cytosolic GS cDNA (Cantón et al., 1993, Plant Mol Biol 22: 819-828) under control of the cauliflower mosaic virus 35S promoter and the *nos* termination sequence defining a *Hind III DNA*-cassette of 2.1 kb (see Material and methods section). T-DNA in the plasmid binary system also contained *nptII* as a selectable marker. Kanamycin-resistant cultures were selected and plants regenerated using standard protocols.

Pine GS1 sequences in the genome of transformed poplar plants. To determine the presence of the GS1 transgene in the genome of kanamycin-resistant poplar plants, total DNA was isolated from leaves and two molecular approaches were conducted in parallel: Southern blotting and PCR amplification. For Southern blotting, genomic DNA from independently transformed poplar transgenic lines was digested with *HindIII*, restriction fragments separated on an agarose gel, and probed with radiolabeled 1.4 kb GS1 cDNA in Southern blots. A hybridization signal corresponding to the 2.1 kb *HindIII*-fragment of the chimeric construct (Figure 1) was detected in all the kanamycin-resistant plants analyzed. No hybridization with labeled pine GS cDNA was detected

in digests of genomic DNA of control, non-transgenic plants.

To further demonstrate the presence of pine sequences in the poplar genome, the DNA of four transforming lines was selected for PCR analysis. Specific amplification was undetectable in control plants whereas an amplification product of the expected size (538 bp) was obtained using DNA from the transformants as a template. The above data clearly indicate the presence of the introduced GS sequences in the selected transgenic clones. The copy number of the introduced gene was estimated by Southern blot analysis; four transgenic lines containing a single copy of the transgene per genome were selected for molecular and biochemical characterization.

Molecular analysis of pine GS1 expression in transgenic poplar. We next examined whether or not the introduced pine GS1 gene is expressed in transgenic poplar. Total RNA was isolated from the selected transgenic poplar and controls, separated on formaldehyde gels and blotted onto Nytran filters. Northern blots probed with radiolabeled pine GS1 cDNA revealed expression of the pine GS message in all transgenic lines. No message was detected among total RNA isolated from non-transformed controls. As an internal control, the coding sequence for the highly conserved, constitutively expressed mitochondrial ß-ATP synthase gene (1.25 kb) (Boutry and Chua, 1985, EMBO J 4:2159-2165) was labelled with ^{32}P and used as a probe. Detection of ß-ATP synthase gene expression in both controls and transgenics indicates appropriate hybridization conditions and equivalent loading of RNA gels.

GS protein levels were studied to further characterize the expression of the transgene. Extracts of total proteins were prepared from whole leaves of control poplar and leaves of transgenic lines, separated on SDS-PAGE, and immunoblots developed using polyclonal

antibodies raised against the recombinant pine GS1 (Cantón et al., 1996, FEBS Lett 393: 205-210). In control, non-transformed leaves, the major GS polypeptide detected corresponds to the 45 kD chloroplastic GS2. However, in leaves of four independent transformed lines, a second major GS polypeptide is detected at 41 kD, which corresponds in size to the introduced pine GS1. As an additional control, total protein extracts from pine cotyledons were also assessed and showed a single GS polypeptide corresponding to the 41 kD GS1.

To confirm these data, protein extracts from tissues enriched in either photosynthetic (leaf tip) or vascular tissues (petioles) of control and transgenic poplar were subjected to western blotting analysis. It is clear that the major GS polypeptide in vascular bundles of non-transformed poplar is the 40 kD GS1, whereas in the same tissue transgenic poplar contains the endogenous 40 kD GS1 and an additional polypeptide corresponding to the 41 kD pine cytosolic GS1 polypeptide. Moreover, in photosynthetic tissues of non-transgenic controls the major polypeptide detected is a 45 kD polypeptide corresponding to the poplar GS2, while in photosynthetic tissues of transgenic poplar lines, an additional major GS polypeptide is detected at 41 kD corresponding to the pine cytosolic GS1. These data suggest that cytosolic pine GS is expressed in both non-photosynthetic and photosynthetic poplar cells.

To confirm the ectopic expression of the cytosolic GS polypeptide in transgenic photosynthetic cells, protein was extracted from control and transgenic leaves in which midribs were removed to minimize the presence of vascular elements expressing endogenous cytosolic GS. The western blot analysis of these extracts showed that only GS2 polypeptide is detected in control plants, whereas both chloroplastic GS2 and pine cytosolic GS polypeptides are present in the extract prepared from transgenic poplar leaves.

Example 2
Phenotypic Characterization of Transgenic Poplar

The example describes the phenotypic characteristics of poplar transformed with a synthetic glutamine synthetase gene.

Materials and Methods

The plant material. The plant material used was a hybrid poplar (Populus tremula X P. alba, clone INRA 717-1B4, Leple JC et al, 1992, Plant Cell Reports 11: 137-141). The transformation and expression of pine GS gene in transgenic poplar is described in Example 1. Regenerated plants were grown without supplemental nitrogen or other amendments.

Protein extraction. 0.4 g of leaves from each development stages of each line were ground in protein extraction buffer (50 mM Tris-HCl pH 8.0 , 2 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol (v/v)). The extracts were centrifuged at 22,000 x g, and the supernatants were used for GS activity and protein content determinations.

GS activity determination. The glutathione synthetase activity was assayed by following established (Canovas et al, 1991, Planta 185: 372-378) with 3 replicates. The differences between the transgenic and non-transgenic plants were statistically analyzed with T-test. The differences between the transgenic lines were statistically analyzed with variance analysis.

Total soluble protein content determination. The protein content was determined by the method described by Bradford (1976, Anal Biochem 72: 248-254) using bovine serum albumin as standard. Three replicates were used.

Chlorophyll content determination. 0.5 g of fully-expanded leaves at each development stage of transgenic lines and controls were ground in 80% (v/v) acetone. The total chlorophyll (a + b) contents were determined by spectrophotometrically reading at the

absorbance 664 nm and 647 nm (Grann and Ort, 1984, J Biol Chem 259:14003-14010). 3 replicates were used.

Height measurements. After the regenerated plants were transferred from the culture room into the greenhouse, the height growth measurements were made weekly. A total of 78 transgenic plants were measured (representing 22 independent transformation events) and 5 non-transgenic controls. The growth differences between the transgenic plants and non-transgenic plants were analyzed with T-test. The differences between the transgenic lines were statistically analyzed with variance analysis.

Leaf lengths, widths, numbers, areas and total photosynthesis area measurement and calculation. The measurements of leaf length and width were made on fully-expanded mature leaves, usually to the 4th node counting from the apex down, of the 22 transgenic lines and controls 5 months after the plants were transferred to the greenhouse. A total of 380 leaves derived from the 22 transgenic lines and 20 leaves from the control plants were measured. The leaf numbers (node numbers) were counted 2, 3 and 6 months after the plants were transferred into greenhouse. Leaf numbers of 78 transgenic plants representing 22 transgenic lines and 5 control plants were determined. Leaf area per leaf was calculated according to the length, width and shape of the leaf. The total photosynthetic area per plant was calculated according to the average area per leaf and the average leaf number per plant.

Results

GS activity, protein and chlorophyll contents. The difference in the GS activity between transgenic lines and control plants is statistically significant (Table 1). The GS activity difference among transgenic lines is also statistically significant ($P< 0.001$). Among the transgenic lines, the highest GS activity is 50.95 nkat/gfw, and the lowest is 24.1 nkat/gfw.

The difference in the chlorophyll concentration between transgenic lines and control plants is statistically significant (Table 1). The differences in the chlorophyll contents of the transgenic lines is also statistically significant ($P < 0.001$). The range of chlorophyll concentration of the transgenic lines is from 787.2 ug /gfw to 488.6 ug / gfw.

Table 1. GS activity, protein contents, and chlorophyll contents between transgenic and control poplars after 6 months in the greenhouse.

	GS activity (nkat / gfw)	Protein content (ug / gfw)	Chlorophyll content (ug / gfw)
Control	22.2	3846.7	484.2
Average of transgenics	36.8	5132.5	586.1
Transgenics > control	14.6 (65.8%)	1285.8 (33.4%)	101.9 (21.0 %)
P	***	***	***

***: $P < 0.001$
22 transgenic lines and control plants were assayed with 3 replicates.

The difference in the of soluble protein content between the transgenic lines and control plants is statistically significant (Table 1). The differences in the total soluble protein contents among transgenic lines is also statistically significant ($P< 0.001$). Among the transgenic lines, the highest total soluble protein content is 7069.2 ug/gfw and the lowest is 3845.7 ug/gfw.

Height growth of transgenic and control plants. Two months after the plants transferred from the culture room to the greenhouse, the average height of the 22 transgenic lines was 76% higher than the control plants (Figure 2). During the first 5 months in the greenhouse, all 22 transgenic lines grew taller than controls. The difference between the transgenic lines and the control plants are statistically significant (Table 2).

Table 2. Net Height Growth of Transgenic and Control Poplars.

Age		Average Height	P	Transgenics > Controls
1 Month	Transgenics	16.4 cm	**	5.5 cm (50.5%)
	Controls	10.9 cm		
3 Months	Transgenics	82.7 cm	***	20.1 cm (32.0%)
	Controls	62.7 cm		
5 Months	Transgenics	120.2 cm	**	19.8 cm (19.7%)
	Controls	100.4 cm		

***: $P < 0.001$; **: $P < 0.01$
A total of 5 controls and 78 transgenic transgenic plants represnting 22 transgenic lines were measured.

After the first month in the greenhouse, the heights of the transgenic plants range from 19.9 cm to 13.1 cm. The tallest transgenic line is 3.5 cm (21.3%) taller than the average height of transgenic plants, and 9.0 cm (82.6%) taller than the average height of non-transgenic control plants. After 3 months in greenhouse, the heights of the transgenic plants range from 93.3 cm to 69.0 cm. The tallest transgenic line is 10.6 cm (12.8%) taller than average height of transgenic plants, and 30.6 cm (48.8%) taller than average height of non-transgenic control plants. After 5 months in greenhouse, the tallest transgenic line is 133.2 cm, which is 13.0 cm (10.8%) taller than average height of transgenic plants, and 32.8 cm (32.7%) taller than average height of the control plants.

Two weeks after the plants were transferred from culture room to greenhouse, weekly height measurements were made up to 6 months. The net growth was calculated, and graphed versus days the plants have been in greenhouse (Figure 3). During the first 4 months, both the transgenic and control plants showed faster growth compared with growth after 4 months. However, the transgenic plants grew faster than controls

at all times during the 6 month greenhouse period.

Average leaf lengths, widths and areas of transgenic and control plants. The leaf lengths and widths were measured on 5 month old plants. The area of the leaves was calculated according to the leaf shapes. The results showed that the differences in leaf lengths, widths and areas between the transgenic and control plants are statistically significant, and the leaf areas of transgenic plants is 10.0 cm^2 (25.4%) larger than those of the control plants (Table 3). The area of the largest leaves from the transgenic plants is 61.2 cm^2, which is 46.1% larger than control plant leaves.

Table 3. Average leaf lengths, widths and areas per leaf of transgenic and control plants.

		Average	P	Transgenics > Controls
Length	Transgenics	12.2 cm	*	1.7 cm (16.5%)
	Controls	10.5 cm		
Width	Transgenics	8.6 cm	*	0.7 cm (8.9%)
	Controls	7.9 cm		
Areas	Transgenics	51.9 cm^2	*	10.0 cm^2 (25.4%)
	Controls	41.9 cm^2		

*: $P < 0.05$.
The measurements of leaf length and width were made on fully-expanded mature leaves of 22 transgenic lines and controls 5 months after the plants were transferred to the greenhouse. A total of 380 leaves derived from 22 transgenic lines and 20 leaves from control plants were measured.

Average leaf number per plant of transgenic and control plants. At 2, 3 and 6 months, the leaf numbers (node number) were determined. The results showed that differences in leaf number between transgenic and control plants are always statistically significant (Table 4). At 2 months, the highest leaf number in a transgenic line was 28.7 leaves per plant, 10.7 leaves (59.4%) more than the leaf number of control plants. At 3 months, the highest leaf number of a transgenic line is 38.0 leaves,

which is 10.5 (38.2%) more than the leaf number of controls. At 6 months, the tallest transgenic line had 49.3 leaves per plant in average, which is 6.3 (14.7%) more than the average leaf number of the control plants.

Table 4. Average number of leaves per transgenic and control plants.

		Leaf Number	P	Transgenics > Controls
2 months	Transgenics	25.3	***	7.3 (40.6 %)
	Controls	18.0		
3 months	Transgenics	35.6	**	8.1 (29.5 %)
	Controls	27.5		
6 months	Transgenics	48.0	**	5.0 (11.6 %)
	Controls	43.0		

***: P< 0.001; **: P< 0.01.
The leaf numbers (node numbers) were counted 2, 3 and 6 months after plants transferred into greenhouse. The leaf numbers of 78 transgenic plants representing 22 transgenic lines and 5 control plants were counted.

Total photosynthetic areas per plant of transgenic and control plants. Six months after the plants were transferred to the greenhouse, the average photosynthetic area per plant was calculated according to the average area per leaf and the average leaf number of per plant. The results showed that the average photosynthetic area per transgenic plant and per control plant were 2491.2 cm^2 and 1801.2 cm^2, respectively. The transgenic plants have 695.5 cm^2 (38.3%) more photosynthetic area per plants than control plants.

The present invention is not limited to the embodiments described and exemplified above, but is capable of variation and modification without departure from the scope of the appended claims.

What is claimed is:

1. A plant expression cassette, which comprises a glutamine synthetase gene coding sequence operably linked to a promoter.

2. The expression cassette of claim 1, wherein the glutamine synthetase coding sequence is from a gymnosperm.

3. The expression cassette of claim 2, wherein the glutamine synthetase coding sequence is from the genus *Pinus*.

4. The expression cassette of claim 3, wherein the glutamate synthetase coding sequence is from *Pinus sylvestris*.

5. The expression cassette of claim 2, in which the promoter is the cauliflower mosaic virus 35S promoter.

6. The expression cassette of claim 5, which further comprises the NOS terminator sequence operably linked to the glutamine synthetase coding sequence.

7. The expression cassette of claim 1, which is contains a nucleic acid sequence selected from the group consisting of:

A.) a nucleic acid sequence that is at least 70% identical to Genbank Accession No. X69822;

B.) a nucleic acid sequence that encodes a protein that is at least 70% similar to Genbank Accession No. X69822;

C.) a nucleic acid sequence that hybridizes to Genbank Accession No. X69822 at moderate stringency; and

D.) a nucleic acid sequence that is Genbank Accession No. X69822.

8. A vector, comprising the expression cassette of claim 1.

9. The vector of claim 8, which is an *Agrobacterium* binary vector.

10. The vector of claim 9, wherein the vector is pBIN19.

11. The vector of claim 10, which further comprises the neomycin phosphotransferase II coding sequence.

12. A method of producing a plant with improved nitrogen metabolism by transforming *in vitro* said plant with the expression cassette of claim 1.

13. The method of claim 12, wherein the plant is a woody perennial.

14. The method of claim 13, wherein the plant is in the family *Salicaceae*.

15. The method of claim 14, wherein the plant is in the genus *Populus*.

16. The method of claim 15, wherein the plant is the hybrid *Populus tremula* X *P. alba*.

17. The method of claim 16, wherein the plant is clone INRA 717 1-B4 of hybrid *Populus tremula* X *P. alba*.

18. The method of claim 12, wherein the transformation step uses the *Agrobacterium tumifaciens* method.

19. The method of claim 13, wherein the transformation step further uses the vector of claim 9.

20. A transgenic plant produced by the method of claim 12.

21. A reproductive unit from the transgenic plant of claim 15.

22. A cell from the transgenic plant of claim 21.

23. A transgenic plant with an improved nitrogen metabolism, which is a woody perennial and comprises at least one transgene that comprises the coding sequence of a glutamine synthetase gene.

24. The transgenic plant of claim 23, wherein the glutamine synthetase gene is from a gymnosperm.

25. The transgenic plant of claim 24, wherein the glutamine synthetase genes is from *Pinus sylvestris*.

26. The transgenic plant of claim 25, wherein the glutamine synthetase coding sequence is Genbank Accession No. X69822.

27. The transgenic plant of claim 17, which is from the family Salicaceae.

28. The transgenic plant of claim 23, which is from the genus *Populus*.

29. The transgenic plant of claim 28, which is a hybrid of *Populus tremula* X *Populus alba*.

30. The transgenic plant of claim 29, which is clone INRA 717 1-B4 of the hybrid *Populus tremula* X *Populus alba*.

31. A reproductive unit from the transgenic plant of claim 23.

32. A transgenic plant, which is a woody perennial, and exhibits a growth rate that is statistically significantly greater at the P=0.05 level than that of equivalent untransformed plants.

33. The transgenic plant of claim 32, which additionally exhibits a protein concentration in gram per gram fresh weight in the mature leaves that is statistically significantly greater at the P=0.05 level than that of mature leaves in equivalent untransformed plants.

34. The transgenic plant of claim 33, which additionally exhibits a chlorophyll concentration in gram per gram fresh weight in the mature leaves that is statistically significantly greater at the P=0.05 level that of mature leaves in equivalent untransformed plants.

35. The transgenic plant of claim 34, which additionally exhibits a mature leaf area in cm^2 per leaf that is statistically significantly greater at the P=0.05 level than that of mature leaves in equivalent untransformed plants.

36. The transgenic plant of claim 32, which is from the family Salicaceae.

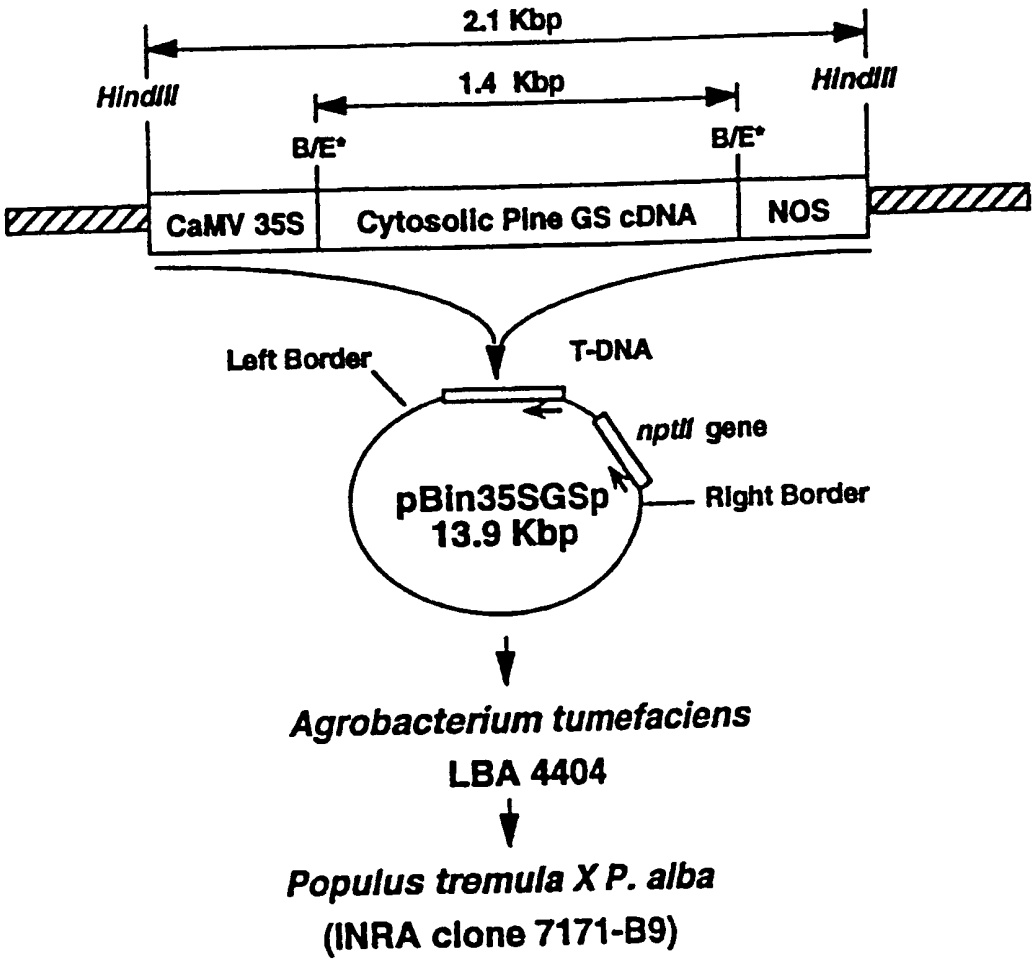
37. The transgenic plant of claim 36, which is from the genus *Populus*.

38. The transgenic plant of claim 37, which is a hybrid of *Populus tremula* X *Populus alba*.

39. The transgenic plant of claim 38, which is clone INRA 717 1-B4 of the hybrid *Populus tremula* X *Populus alba*.

40. A reproductive unit from the transgenic plant of claim 32.

Figure 1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1.4 Kbp
HindIII
B/E*
B/E*
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NOS
Left Border
T-DNA
nptII gene
pBin35SGSp
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Right Border
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LBA 4404
Populus tremula X P. alba
(INRA clone 7171-B9)

Figure 2

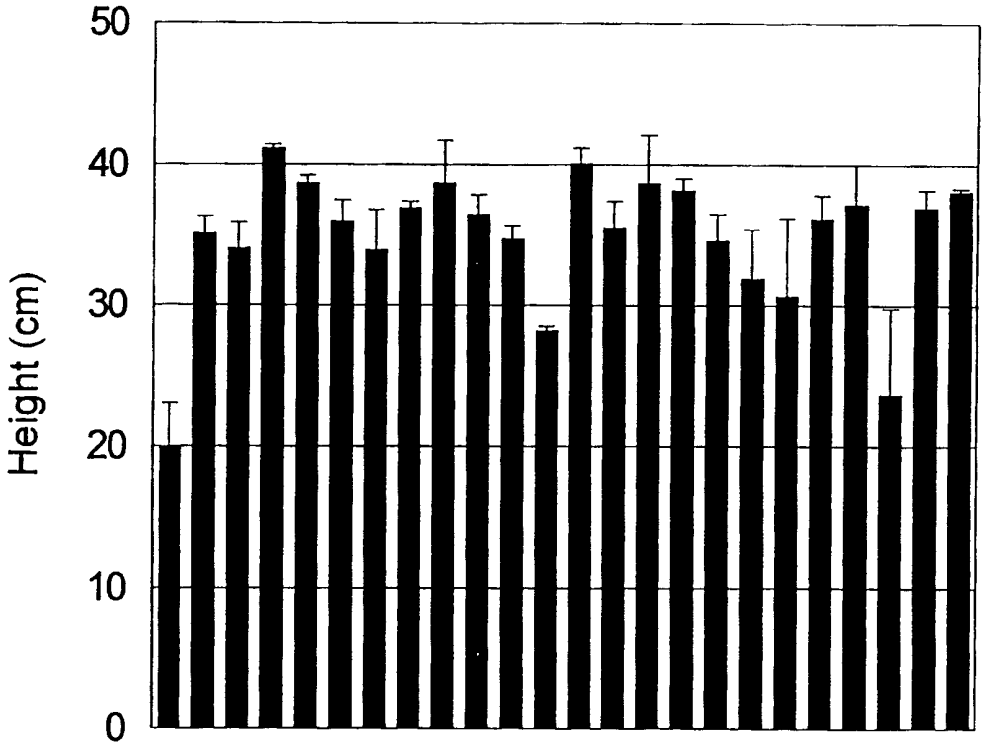
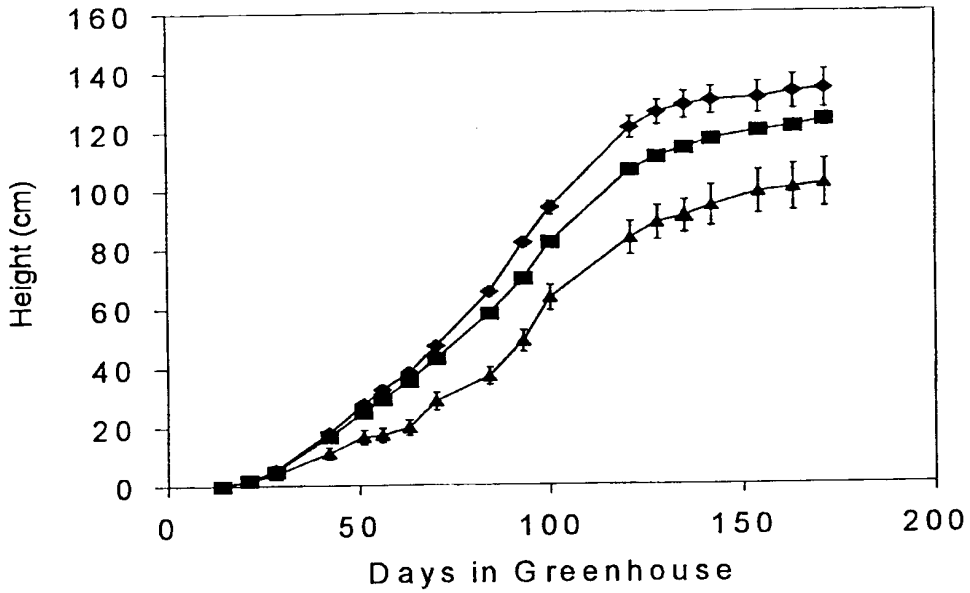


Figure 3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60
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Height (cm)
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50
100
150
200
Days in Greenhouse

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/18267

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/82, 5/04, 15/29; A01H 4/00, 5/00, 5/10

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 468, 410, 419, 320.1; 536/23.1, 23.2, 23.6; 800/277, 290, 295, 319, 323

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, EAST, MEDLINE, AGRICOLA, BIOSIS, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	TEMPLE et al. Modulation of Glutamine Synthetase Gene Expression in Tobacco by the Introduction of an Alfalfa Glutamine Synthatase Gene in Sense and Antisense Orientation: Molecular and Biochemical Analysis. Mol. Gen. Genet. 1993, Vol. 236, pages,315-325, see entire document.	1 ----- 1-40
X --- Y	PARSONS et al. Transformation of Poplar by Agrobacterium Tumefaciens. Bio/Technology. 1986. Vol. 4, pages 533-536, see entire document.	32-37 ------- 1-40
Y	LIN et al. A cDNA Sequence Encoding Glutamine Synthetase is Preferentially Expressed in Nodules of Vigna Aconitifolia. Plant Physiol. January 1995, Vol. 107, pages 279-280, see GENBANK/ Accession No. M94765.	1-40

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 NOVEMBER 1999	23 DEC 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer OUSAMA M-FAIZ ZAGHMOUT Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

Intern ..ional application No.
PCT/US99/18267

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 468, 410, 419, 320.1; 536/23.1, 23.2, 23.6; 800/277, 290, 295, 319, 323

High-frequency transformation of cottonwoods (genus *Populus*) by *Agrobacterium rhizogenes*

Kyung-Hwan Han, Milton P. Gordon, and Steven H. Strauss

Abstract: Many species of *Populus*, particularly cottonwoods of sections Aigeiros and Tacamahaca, remain recalcitrant to genetic transformation. We demonstrate that transgenic trees can be readily produced in several poplar genotypes using wild-type *Agrobacterium rhizogenes*. Hairy roots were produced in a variety of clones that included *Populus trichocarpa* Torr. & A. Gray, *Populus deltoides* Bartr. ex Marsh., *P. trichocarpa* × *P. deltoides* hybrids, and *Populus alba* L. × *Populus grandidentata* Michx., some of which were otherwise recalcitrant to regeneration or transformation. The frequency of hairy roots ranged from 17 to 92%; nearly all hairy roots could be regenerated into transgenic plants. Among 18 transgenic lines that were confirmed by Southern analysis and grown into small trees, 5 showed severe dwarfism and other phenotypic abnormalities, while the majority grew normally. *Agrobacterium rhizogenes* appears to be an effective means for transformation of diverse poplar genotypes and may be useful in genotypes where disarmed strains of *Agrobacterium* or regeneration protocols are inadequate to produce transgenic plants.

Résumé : Plusieurs espèces du genre *Populus*, et plus particulièrement celles des sections Aigeiros et Tacamahaca, demeurent réfractaires à la transformation génétique. Les auteurs démontrent que des arbres transgéniques peuvent être obtenus facilement pour plusieurs génotypes de peuplier à l'aide d'une souche sauvage de *Agrobacterium rhizogenes*. Des racines avec poils absorbants ont été obtenues pour un nombre important de clones représentatifs des espèces *Populus trichocarpa* Torr. & A. Gray et *Populus deltoides* Bartr. ex Marsh., ainsi que des hybrides de *P. trichocarpa* × *P. deltoides* et de *Populus alba* L. × *Populus grandidentata* Michx., dont certains étaient réfractaires à la régénération ou à la transformation. La fréquence à laquelle des racines avec poils absorbants furent obtenues variait de 17 à 92% et presque toutes les lignées de racines avec poils absorbants pouvaient être régénérées en plantes transgéniques. Parmi les 18 lignées transgéniques qui furent confirmées par analyse de type Southern et qui se sont développées jusqu'au stade de petit arbre, 5 ont affiché un nanisme sévère ainsi que d'autres anormalités phénotypiques, alors que la majorité des lignées se sont développées normalement. L'utilisation de *A. rhizogenes* apparaît donc être un moyen efficace pour transformer divers génotypes de peuplier. Cette stratégie pourrait être utile pour la production de plantes transgéniques dans le cas des génotypes pour lesquels la transformation par des souches désarmées de *Agrobacterium* ou les protocoles de régénération s'avèrent inadéquats. [Traduit par la Rédaction]

Introduction

Poplar species are important for biomass, pulp and paper, and solid wood products, and commercial plantation area is growing rapidly in the United States. Genetic engineering can complement conventional breeding efforts by providing means for insertion of genes that confer desired traits not readily available in sexually accessible gene pools. In contrast with sexual breeding, it allows new genes to be added while the desired genotypes of clones are preserved and can therefore reduce the time required to produce an elite line. For genetic engineering to be commercially viable, transformation systems are needed that can efficiently produce transgenic trees from diverse genotypes.

Received April 2, 1996. Accepted October 21, 1996.

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Poplars have been transformed in many laboratories. However, the majority of work has been confined to a few model hybrids of aspen (reviewed in Jouanin et al. 1993). Cottonwoods of sections Aigeiros and Tacamahaca are usually recalcitrant to regeneration and transformation. There has been only one published report of transgenic cottonwoods (*Populus trichocarpa* Torr. & A. Gray × *Populus deltoides* Bartr. ex Marsh.) established in soil (De Block 1990). One possible explanation for its recalcitrance to transformation is the lack of an efficient regeneration system (De Block 1990), which often requires customizing regeneration conditions to specific genotypes (Han et al. 1995) to promote cell competence for regeneration (Coleman and Ernst 1990). An alternative to genotype-specific manipulation may be to take advantage of the morphogenetic effects of genes transferred by *Agrobacterium rhizogenes*.

The soil bacteria *Agrobacterium tumefaciens* and *A. rhizogenes* are the two species widely used to transform higher plants, including poplars. Both systems rely on a similar transformation mechanism. However, an important difference between the two species is that transgenic plants can be regenerated from roots induced by wild-type *A. rhizogenes*, while plant cells transformed with *A. tumefaciens* carrying wild-type Ti (tumor-inducing) plasmids cannot be regenerated into plants. Plants regenerated from Ri (root-inducing)-transformed roots,

however, display the "hairy-root syndrome" (described in Tepfer 1984) caused by the T-DNA (transferred DNA)-borne genes (Schmulling et al. 1988; Gaudin et al. 1994). Transformation with a disarmed *A. tumefaciens* strain produces transgenic plants without such abnormal phenotypes; however, with disarmed *A. tumefaciens* species a precise regeneration–selection system must be developed for each plant species or genotype. Although efficiency varies widely, *A. rhizogenes* has been used to produce transgenic trees in *Eucalyptus* (Macrae and van Staden 1993), *Larix* (Huang et al. 1991; Shin et al. 1994), *Malus* (Lambert and Tepfer 1992), *Populus* (Brasileiro et al. 1991; Charest et al. 1992; Pythoud et al. 1987), and *Robinia* (Han et al. 1993). Pythoud et al. (1987) reported genetic transformation of hybrid cottonwood (*P. trichocarpa* × *P. deltoides*) clone H11 using *A. rhizogenes*. They were able to regenerate shoots from callus tissues derived from the hairy roots, but did not propagate the shoots in soil or report on shoot morphology.

The abnormal growth often observed in plants transformed with *A. rhizogenes* may be used to advantage in some instances. Careful phenotypic testing of transgenic trees may identify transformants with increased growth rates and larger root masses (Han et al. 1993; Tepfer 1984). The adaptive significance of extensive root development may include improved anchorage, increased interactions with soil microorganisms, and stronger drought tolerance. Apple trees infected with *A. rhizogenes* withstood drought better than uninfected trees (Moore et al. 1979). The greater production of root exudates and water uptake expected from large root systems may increase soil remediation by direct uptake and plant and microbial degradation.

In the present study, we describe an efficient transformation system for a recalcitrant but economically important kind of hybrid cottonwood (*P. trichocarpa* × *P. deltoides*). The Ri-transformed poplar trees were regenerated and established in soil, stable incorporation of T-DNA genes into the poplar genome is demonstrated by Southern blot analysis, and phenotypic effects of the expression of Ri T-DNA genes in the transgenic trees are documented.

Materials and methods

Bacterial strains

Four bacterial strains were used. *Agrobacterium rhizogenes* R1000 has the chromosomal background of *A. tumefaciens* strain C58 into which the Ri plasmid of pRiA4b has been conjugated (White et al. 1985). The strain R1240 is disarmed by removal of the core T_L- and T_R-DNA (White et al. 1985). Strain R1600 is similar to R1000, but carries the cosmid pTVK291 (Jin et al. 1987), which contains the virulence region from the "supervirulent" plasmid, pTiBo542 (Hood et al. 1984). R1601 is the same as R1600 except that the neomycin phosphotransferase gene (*nptII*) from the bacterial transposon Tn*5* is inserted into the *Hin*dIII fragment 21 of the Ri plasmid (Sinkar et al. 1988*a*). The bacteria were grown overnight at 28°C on Luria–Bertani medium (LB; contains 10 g/L Bacto-tryptone, 5 g/L Bacto-yeast extract, and 10 g/L NaCl, adjusted to pH 7.0). The bacteria were then collected by centrifugation and resuspended in LB medium supplemented with 50 mM acetosyringone and 0.1 M galactose.

Tissue culture and transformation

The protocols described for transformation (Han et al. 1993, 1994) and for tissue culture (Han et al. 1995) were used in this study, with minor modifications. *Agrobacterium rhizogenes* strains used as transformation agents are listed in Table 1. Greenwood stems (about 5 mm in diameter) were sterilized in 30% Clorox solution for 15 min followed by five washes with sterile distilled water. Internodal stem discs (5 mm thick) were cut and soaked for several minutes in an overnight culture of *A. rhizogenes* (A_{600} = 1.0) and placed on solid Murashige and Skoog (MS) medium (Murashige and Skoog 1962) without growth regulators (MSO). After 3 days of co-cultivation, the explants were washed in sterile distilled water three times and transferred to either MSO or MS supplemented with 1 µM indolebutyric acid (IBA) containing 300 mg/L Timentin® (SmithKline Beecham Pharmaceuticals, Philadelphia, Pa.) to kill residual bacteria. The tissue was maintained on the same medium for at least 3 weekly subculture periods for hairy root induction. For shoot regeneration, hairy roots from the clone 53-246 induced by the strain R1601 were cultured on shoot induction medium (MS + 5 µM 6-benzylaminopurine + 10 µM α-naphthaleneacetic acid) with 100 mg/L kanamycin. Shoots regenerated from stem internodes of clone 53-246 that were not treated with *Agrobacterium* were used as negative controls throughout the experiments.

Growth of transgenic poplars

Shoots regenerated from hairy roots were multiplied and rooted in a Magenta G-7 box (Sigma Co.) containing 50 mL of shoot induction medium with kanamycin. Four ramets per independent transgenic clone were potted (25 cm diameter pot) as in Han et al. (1990) and grown in a greenhouse. Measurements were taken after 5 months of growth; trees were then pruned.

Southern blot analysis

Total plant DNA was isolated from young leaves harvested from potted plants, as in Bradshaw and Stettler (1993). Isolated DNA (3 µg/lane) was digested with *Hin*dIII and transferred to a Zeta-probe blotting membrane by DNA alkaline blotting as specified by the manufacturer (Bio-Rad). The blots were hybridized in roller bottles (Robins Scientific) with approximately 5×10^6 cpm/mL (1 cpm (count per minute) = 0.0167 Bq) ^{32}P-labeled probe in 0.5 M Na_2HPO_4, pH 7.2, 7% SDS, 10 mM EDTA, and 0.5% nonfat dry milk at 65°C overnight and washed according to Church and Gilbert (1984). Autoradiography was done at –70°C with a single intensifying screen. For probes we used either the *Hin*dIII fragment 16 (T_L-DNA right junction fragment; Han et al. 1993), T_L-DNA (pFW302; White et al. 1985), or T_R-DNA (pFW41; Huffman et al. 1984).

Statistical analysis

All data were processed using analysis of variance (ANOVA) from the GLM procedure in SAS (SAS Institute Inc. 1990). The data for each clone were separated by Duncan's multiple range test where ANOVA indicated statistical significance.

Results

Transformation

Hairy roots started to form on the cut surface of stem discs 7 days after inoculation with *A. rhizogenes*. These roots differed from normal roots in the following ways: (1) roots were initiated in the presence of little or no auxin in the culture medium; (2) hairy roots arose on the cut surface from cells that were transformed with *A. rhizogenes*, while normal roots were induced on the periphery of the stem segments in response to exogenously supplied auxin that diffused into the stem tissue from the medium (Figs. 1A, 1B, and 1C); and (3) hairy roots developed a large number of root hairs and lateral branches (Figs. 1A and 1B).

Hairy root formation frequency varied from 17 to 92%, depending on plant genotype (Table 1). Infection with the wild-type strain R1000 transformed the hybrid cottonwood clone

Fig. 1. (A) Hairy root formation on stem segments after 1–3 weeks of inoculation. (B) Hairy root showing a unique morphology such as branching and abundant root hairs. (C) Normal adventitious roots induced by auxin treatment, showing no significant branching or root hairs. (D) Shoot regeneration from a hairy root. (E) Transgenic shoot cultures have tendency to produce aerial root mass. (F) Transgenic tree with altered phenotype such as dark green, wrinkled leaves. (G) Transgenic trees with normal phenotype.

24-305 (17%), but the infection with the supervirulent strain R1600 substantially increased transformation frequency (92%). The disarmed strain R1240 produced no hairy roots after infection. *Populus deltoides* clone ILL129, which did not respond to ordinary tissue culture manipulations for regeneration, produced hairy roots from 19% of explants. Spontaneous shoot regeneration (7%) was also observed with this clone when inoculated with R1601.

The hairy root tissues (longer than 2 cm) induced by R1601 from the clone 53-246 were transferred to shoot induction medium. Shoot regeneration was observed on roots 20–35 days later (Fig. 1D). Nineteen of 75 hairy roots (25%) produced transgenic shoots in the presence of 100 mg/L kanamycin. All of the resulting shoots were successfully rooted in either MSO or MS medium supplemented with 1 μM IBA in the presence of 100 mg/L kanamycin.

Phenotypic consequences of transformation

Hairy root regenerants easily produced aerial roots in tissue culture (Fig. 1E), and their roots showed plagiotropism in soil. They tended to form densely packed, horizontally radiating roots near the surface of the soil. About 10% of all transgenic

Table 1. Hairy root formation frequency.

Bacterial strains*	Plant genotype†	No. of explants	Explants producing hairy roots (%)‡
R1000	TD: 24-305	60	17
R1240	TD: 24-305	60	0
R1600	TD: 24-305	60	92
R1601	T: 93-968	41	22
R1601	D: ILL129	42	19
R1601	TD: 53-242	39	23
R1601	TD: 53-246	46	44
R1601	TD: H11	33	85
R1601	AG: NC-5339	48	69

*R1000, *A. tumefaciens* strain C58 + pRiA4b; R1240, disarmed R1000; R1600, R1000 + pTVK291 (carries the virulence region of a supervirulent plasmid pTiBo542); R1601, R1600 + *nptII* gene.

†T, *P. trichocarpa*; D, *P. deltoides*; TD, *P. trichocarpa* × *P. deltoides*; AG, *Populus alba* L. × *P. grandidentata* Michx. Clones 53-246, 93-968, and ILL129 were obtained from Dr. Toby Bradshaw at University of Washington, Seattle, Wash., U.S.A.; H11 and NC-5339, from Brent McCown at University of Wisconsin, Madison, Wis., U.S.A.; and 24-305, from Dr. Brian Stanton at the James River Corporation, Camas, Wash.

‡Results from single experiment.

trees had dark green, wrinkled, and thick leaves (Fig. 1F). The wrinkled leaf morphology appeared and disappeared several times during tree development. Six of 18 hairy root regenerants had stunted growth. These dwarfed trees had short internodes and abundant lateral branches. Some of the lateral branches were vinelike, tended to grow horizontally, and grew faster than the apical leader. Applications of 2.5 mM GA_3 (gibberellic acid; Sigma Co.) to the apical buds of these trees resulted in increased shoot growth. Two-thirds of the hairy root regenerants showed apical dominance and were morphologically indistinguishable from the normal plant (Fig. 1G). One regenerant (line 6D9, carrying only T_R-DNA) was significantly taller than normal. All transgenics had fewer branches than did normal plants with the exception of one, whose number was not significantly different from normal (Table 2). In the summer of 1995 we planted these transgenic trees in the field for the study of transgene expression over time.

Southern blot analyses

Southern blot analysis using the right border sequences of T_L-DNA as a probe showed that all four transgenic trees analyzed had multiple insertions of T_L-DNA into the poplar genome (Fig. 2). Of 15 transgenic lines analyzed by Southern blot analyses using either T_L- or T_R-DNA sequences as probes, 13 lines had both T_L- and T_R-DNA sequences, while two (clones 6D9 and R20) had only T_R-DNA sequences. These two clones (6D9 and R20) showed the greatest height growth. Eight transgenic lines carrying both T_L- and T_R-DNA sequences were used in growth measurements. Only 2 of the 8 lines, however, showed stunted growth.

Discussion

The phenotype of hairy root transgenic plants ranges from normal (Huang et al. 1991) through a variety of altered phenotypes, including larger root masses (Han et al. 1993; Tepfer 1984), greater growth rates (Strobel and Nachmias 1985; Strobel et al. 1988), wrinkled leaves (Christy and Sinclair 1992), and increased drought tolerance (Moore et al. 1979). Two-thirds of our transgenic cottonwoods showed no obvious hairy root phenotypes when established in soil, while one-third had severely stunted shoot growth.

Dwarfism was also observed among transgenic tobacco plants overexpressing the *rolA* gene (Dehio et al. 1993) and potato plants overexpressing the *rolC* gene (Schmulling et al. 1993). Leaves of those plants contained 20–60% less GA_1 than control plants. The GA_3 application reduced the severity of phenotypic alteration induced by the *rolA* transgene but not by the *rolC* transgene (Schmulling et al. 1993). Promoter activities of both *rolB* and *rolC* differed from that of *rolA* in transgenic tobacco (Schmulling et al. 1988). In all trees tested, the dwarfism of our cottonwood clones appeared to be reversed by GA_3 application. This suggests that initially they had low gibberellic acid concentrations because of the overexpression of the *rolA* gene. The lack of dwarfism among most of our transformants, even when they contained both T_L- and T_R-DNA sequences, may have resulted from differential transfer or expression of individual *rol* genes among independent transformants.

We observed dark green, wrinkled leaves in about 10% of the transgenic poplars. This phenotype varied in expression during development, with no obvious environmental cue, when the trees were maintained in a greenhouse. The wrinkled leaves tended to appear in the young leaves on the leader but not on the branches. Expression of the *rolA* transgene was responsible for dark green, wrinkled leaves in transgenic potato (Schmulling et al. 1993).

Transgenic trees with normal phenotypes suggest that the *rol* transgenes may have been silenced in those trees after the hairy root formation–regeneration process. Sinkar et al. (1988*b*) observed frequent reversion of hairy root phenotypes in tobacco. Their Southern and Northern blot analyses suggested that the revertant phenotypes were due to the transcriptional inactivation of T-DNA genes. Gene silencing in transgenic plants is widely known (Finnegan and McElroy 1994). Brandle et al. (1995) reported that co-suppression of a transgene was triggered by the common agronomic practice of seedling transplantation in field-grown transgenic tobacco. Several different genetic mechanisms appear to be involved (Matzke and Matzke 1995), often associated with multiple copies of transgenes (Flavell 1994) and DNA methylation.

We report high transformation efficiency for hybrid cottonwood, which has shown recalcitrance in our transformation experiments with *A. tumefaciens*. The main obstacle in transformation appears to be targeting and isolating regenerable cells. Actively dividing cells are more susceptible to *Agrobacterium* infection (Akama et al. 1992), and competence for transformation is associated with the timing of cell division at the wound site (Braun and Mandle 1948). Therefore, transformation frequency may be improved by increasing the number of regenerable cells that are in active cell division. *Agrobacterium rhizogenes* may "naturally" condition the plant cells for transformation–regeneration by providing plant hormones to promote cell division in cells surrounding the wound site. Conditioning may also result from morphogenic potential in hairy root tissues. For example, inoculation of *P. deltoides* clone ILL129 with *A. rhizogenes* strain R1601 resulted in high root

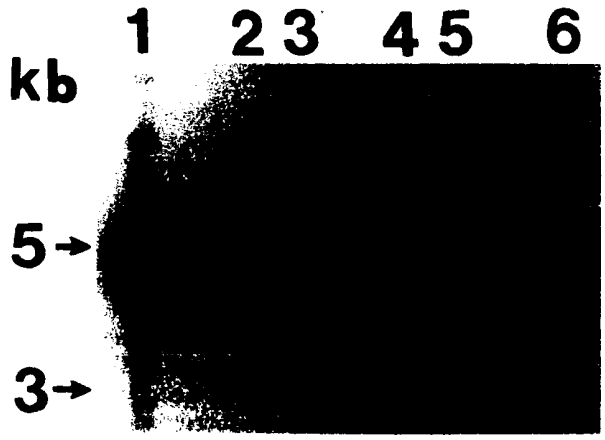
Table 2. Growth of *Agrobacterium rhizogenes* transgenic poplars after 5 months in the greenhouse.

Transgenic clones*	Height (cm)	Stem diam. (mm)	Leaf length (mm)	Leaf width (mm)	No. of leaves	Branch length (mm)	Branch diam. (mm)	No. of branches
6Z7	9*d*	3*e*	49*d*	25*c*	7*f*	0*b*	0*b*	0*c*
6RZ1	10*d*	4*e*	55*d*	28*c*	8*ef*	0*b*	0*b*	0*c*
6D1	11*d*	3*e*	52*d*	23*c*	7*f*	0*b*	0*b*	0*c*
R6-30	13*d*	4*e*	56*d*	35*c*	6*f*	0*b*	0*b*	0*c*
6R24	25*d*	4*e*	73*d*	51*c*	12*e*	0*b*	0*b*	0*c*
6D13	112*c*	9*d*	222*c*	142*ab*	18*d*	230*a*	3.2*a*	2.5*bc*
6R9	131*bc*	11*abcd*	273*ab*	163*ab*	30*ab*	247*a*	2.9*a*	15.7*a*
6D14	131*bc*	10*bcd*	241*abc*	132*b*	25*bc*	195*a*	2.7*a*	3.0*bc*
6D12	134*bc*	11*abcd*	226*bc*	135*b*	27*bc*	188*a*	2.4*a*	3.8*bc*
6R26	137*bc*	10*bcd*	248*abc*	138*ab*	27*bc*	248*a*	3.2*a*	6.0*b*
R6-31	143*b*	11*abcd*	258*abc*	161*ab*	25*bc*	228*a*	3.0*a*	3.5*bc*
6D5	145*b*	10*bcd*	239*abc*	139*ab*	27*bc*	125*ab*	2.1*a*	5.3*b*
R1991	146*b*	11*abcd*	261*abc*	159*ab*	30*ab*	243*a*	2.5*a*	5.5*b*
6R18	147*b*	11*abcd*	239*abc*	136*ab*	28*bc*	168*a*	2.4*a*	3.3*bc*
6D16	152*ab*	12*ab*	261*abc*	160*ab*	24*c*	158*a*	2.2*a*	2.5*bc*
6R25	153*ab*	11*abcd*	265*abc*	155*ab*	29*bc*	223*a*	3.1*a*	4.0*bc*
6R20	154*ab*	12*ab*	277*a*	168*a*	27*bc*	283*a*	3.0*a*	5.0*b*
6D9	173*a*	13*a*	270*abc*	165*ab*	30*ab*	188*a*	2.4*a*	3.5*bc*
Control	139*b*	12*ab*	260*abc*	155*ab*	34*a*	277*a*	3.3*a*	13.7*a*

Note: Means with different letters are significantly different at the 0.01 probability level.

*All transgenic lines were derived from *P. trichocarpa* × *P. deltoides* clone 53-246, and control was nontransgenic shoot regenerated from stem internode of the same clone.

Fig. 2. Southern blot analysis of transgenic poplars. Total poplar DNA was digested with *Hin*dIII, size-fractionated (in kilobases (kb)) on agarose gel by electrophoresis, and transferred to Zeta-probe membrane (Bio-Rad). The blots were hybridized with ^{32}P-labeled *Hin*dIII border fragment (H-16) of T_L-DNA of *A. rhizogenes* R1601 (White et al. 1985). Autoradiograms of blots are shown: lane 1, total DNA (10 ng) of *A. rhizogenes* R1601; lane 2, normal poplar (3 µg); lanes 3–6, transformants 6D1, 6D8, 6D11, and 6R24 (3 µg each), respectively. Each hybridizing band indicates the incorporation of a copy of T_L-DNA.



and shoot regeneration. Without inoculation this particular clone was incapable of regeneration in our tissue culture studies (Han et al. 1995). *Agrobacterium rhizogenes* has been the only vector able to give rise to transformed plants in some recalcitrant tree species (Han et al. 1993; Shin et al. 1994).

There are at least three possible means of taking advantage of *A. rhizogenes* for transformation of recalcitrant species without encountering phenotypic abnormalities. One is to place the *rol* genes under an inducible gene expression system and have them expressed only during the transformation process. A number of systems for inducible gene expression have now been described in plants (Roder et al. 1994; Mett et al. 1996). Another option is to use co-transformation, where two separate T-DNAs contained within a single bacterium are transferred into plant genomes. Binary vectors derived from *A. tumefaciens* can be efficiently transferred into plant cells when they are placed in a virulent strain of *A. rhizogenes*. A high frequency of co-transfer of wild-type and binary vector T-DNAs is generally obtained: 43–60% in alfalfa (Simpson et al. 1986; Sukhapinda et al. 1987), 39–85 % in *Brassica* (De Block and Debrouwer 1991), 86 % in *Stylosanthes* (Manners and Way 1989), and 70–80% in tobacco (Hamill et al. 1987; Hamamoto et al. 1990). As demonstrated in the present study, these hairy roots can often be induced to regenerate into transgenic plants. Under some conditions, the T-DNAs from the binary and wild-type plasmids integrate into unlinked sites and can be segregated during sexual reproduction. Moreover, in a population of co-cultivated cells, not all will be transformed with both the wild-type and binary T-DNA, potentiating their separation during regeneration. For example, when co-transformed tomato tissues were cultured on a medium supplemented with appropriate growth regulators and antibiotics, they produced callus instead of hairy roots, from which phenotypically normal transgenic plants could sometimes be obtained (Shahin et al. 1986). Finally, our results suggest that a useful proportion of transgenic poplar lines produced with *A. rhizogenes* transformation show no discernible phenotypic effect; whatever the mechanism, it may be possible to simply screen

a large number of transgenic lines for those that remain free of abnormalities.

Acknowledgements

We thank Ronglin Wu for his help on the measurement and statistical analyses of growth data, Priscilla Licht for editing the manuscript, and the Tree Genetic Engineering Research Cooperative at Oregon State University and the Occidental Chemical Corporation for financial support.

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